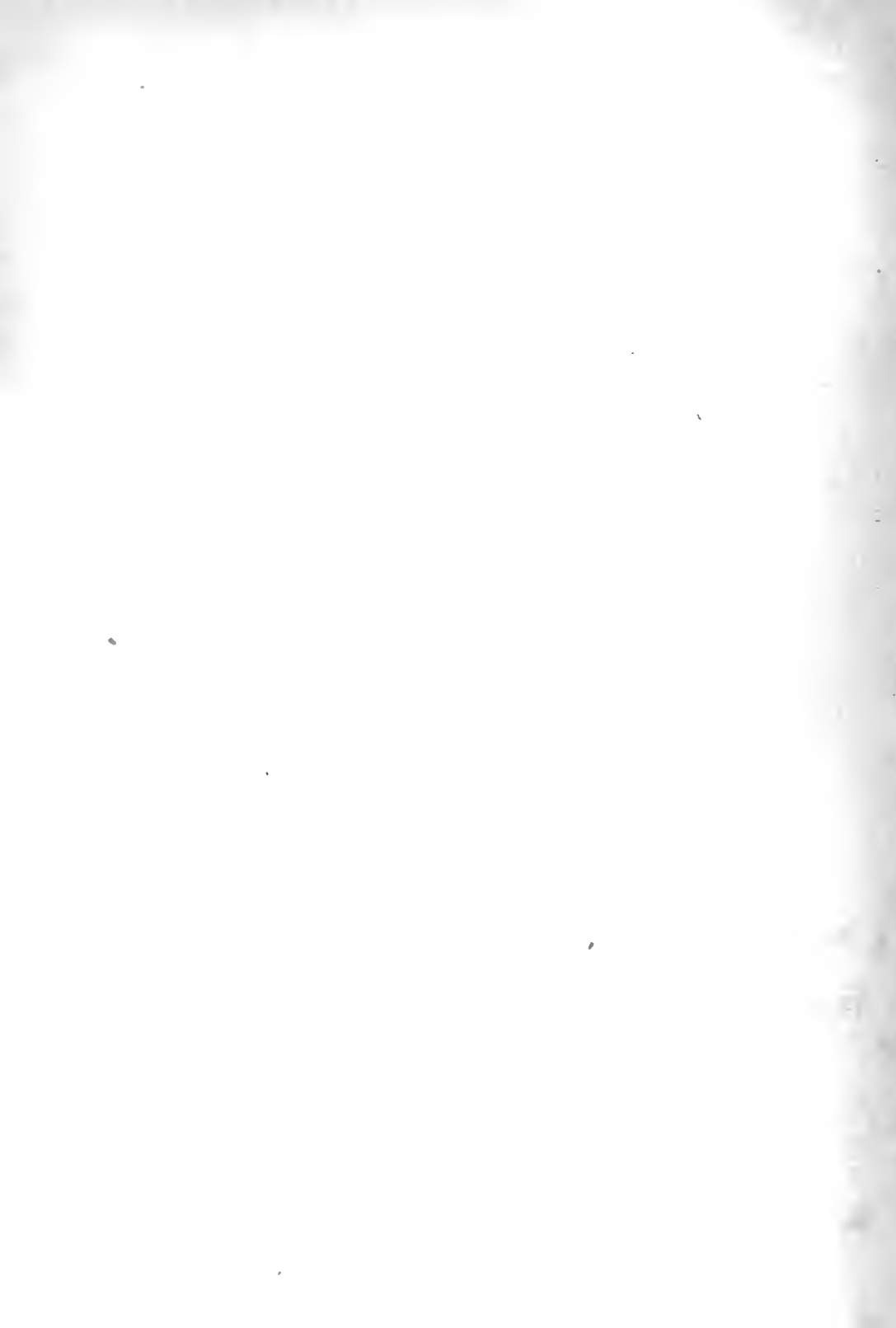




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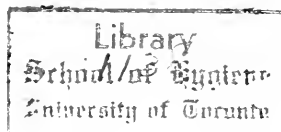
LUDVIG HEKTOEN AND EDWIN O. JORDAN

IN CONJUNCTION WITH

FRANK BILLINGS      F. G. NOVY

W. T. SEDGWICK

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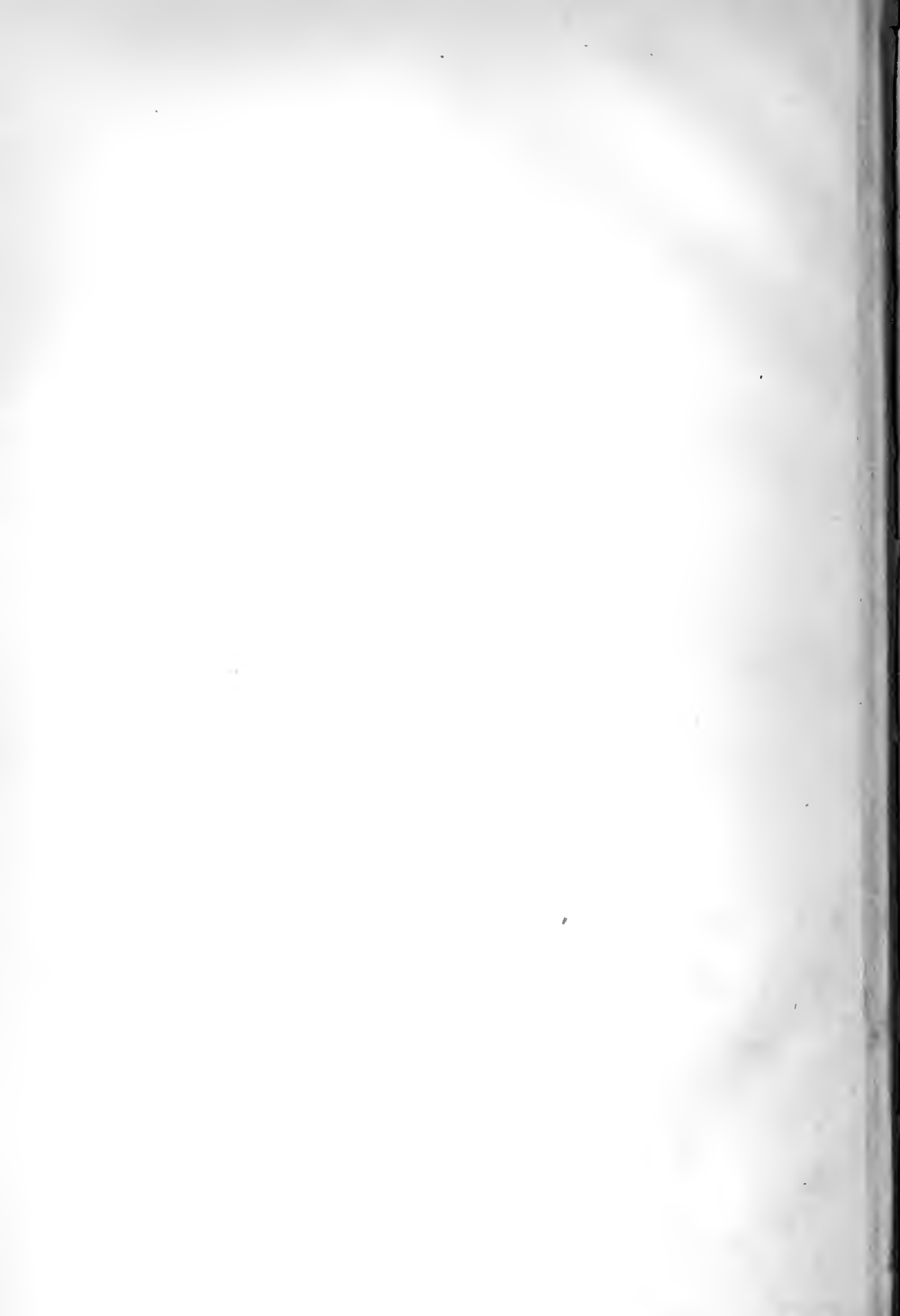
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# *The* Journal of Infectious Diseases

FOUNDED BY THE MEMORIAL INSTITUTE FOR INFECTIOUS DISEASES

VOL. 5

January 30, 1908

No. 1

## A STUDY OF TYPHOID OPSONINS.\*

CHARLES P. CLARK AND J. P. SIMONDS.

(From the Memorial Institute for Infectious Diseases, Chicago.)

THE investigations which have been made in regard to the opsonins arising either during the course of typhoid fever, or subsequent to the inoculation of animals with living or dead cultures of typhoid bacilli, have not led to uniform conclusions. Wright and Douglas<sup>1</sup> found that the typhoid bacillus is highly susceptible to the opsonic action of serum; they class typhoid fever, however, with those diseases which give a constantly low opsonic index.

Leishman, Harrison, Smallman, and Tulloch<sup>2</sup> came to the conclusion that opsonin, if present in typhoid fever, cannot be demonstrated by the technic employed by them.

H. Klien<sup>3</sup> reports the results of a thorough study of the opsonins arising subsequent to the subcutaneous injection of living typhoid bacilli into rabbits. The results which he obtained by the use of Wright's original method of determining the opsonic index would indicate that the index after such injections does not rise very high. Klien, however, placed little confidence in the results obtained by this method,

\* Received for publication December 10, 1907.

<sup>1</sup> *Proc. Roy. Soc.*, 1904, 73, p. 136.

<sup>2</sup> *Jour. Hyg.*, 1905, 5, p. 380.

<sup>3</sup> *Bull. Johns Hopkins Hosp.*, 1907, 18, p. 245.

and on that account he conducted his later examinations on a different method. He determined the point of dilution at which phagocytic action was sufficiently great to give a phagocytic index of 0.5. This degree of dilution was taken to represent the degree of opsonic activity. From his study of typho-opsonins with this method Klien concludes unreservedly that "it has been shown that by immunizing a rabbit against typhoid, opsonins are formed in a considerably high degree."

Klien also found that agglutinins, opsonins, and lysins increase at the same rate after the injections, but that later the opsonins and lysins increase more rapidly than the agglutinins; the rates of increase in the former may be identical; furthermore, that immune opsonin is thermostable, resisting heating at 55-58° C. for 30 minutes, and that normal opsonin is thermolabile but yet does not disappear entirely after exposure to a temperature of 56-58° C. for 30 minutes.

Neufeld and Hüne<sup>1</sup> regard the opsonic action of normal serum as due to amboceptor and complement, which cause not complete death of the bacteria acted upon, but a certain amount of injury which renders them susceptible to phagocytosis; whereas immune opsonins are held to be distinct new substances which resist the action of heat. They acknowledge, however, that sometimes undiluted normal serum after being heated to 56° C. for 30 minutes favors phagocytosis of certain bacteria. But in this case they hold the phenomenon to be unexplained. They found that the serum from a patient with paratyphoid fever and sera of animals immunized with paratyphoid bacilli may contain opsonin, or tropic substances, as they say, but no lysin for paratyphoid bacilli. Such sera may, however, contain lysin for typhoid bacilli.

#### METHOD.

The plan pursued in our investigations is essentially that used by Wright and his followers in the determination of indices for other organisms, and may be considered under the following headings:

*Washed leucocytes.*—These are obtained by collecting in 0.85 per cent sodium-citrate solution blood from the lobule of the ear. The leucocytes and erythrocytes are now precipitated by centrifugal force and subsequently washed once or twice with physiological salt solution. The "cream" is then drawn off and used in the experiment.

<sup>1</sup> *Centralbl. f. Bakt., Abt. 1*, 1908, 38, Ref. p. 28; *Arch. a. d. kais. Gesund.*, 1907, 25, p. 1.



*Bacillary suspension.*—A 24-hour broth culture of the typhoid bacillus is diluted to a moderate cloudiness; otherwise spontaneous phagocytosis will most likely interfere with the results.

Typhoid bacilli even when recently isolated may give considerable spontaneous phagocytosis while, so far as observed, fresh paratyphoid bacilli give practically none at all. Repeated transplantation may render a typhoid strain originally not subject to spontaneous phagocytosis quite markedly phagocytatable without the intervention of serum. The 24-hour broth cultures of typhoid bacilli are much denser than those of paratyphoid bacilli, and we found that, as a rule, dilution of typhoid broth cultures so that the density approximates the undiluted paratyphoid culture obviates spontaneous phagocytosis almost completely. While before dilution there may be spontaneous phagocytosis of from  $\frac{1}{2}$  to 1 bacillus per leucocyte and even more, dilution reduces this to nil or practically nil (0.018, 0.002, etc.). The most useful suspensions are those giving an average phagocytosis of 1.0–1.5 bacillus per leucocyte with heated normal serum; if suspensions of greater density are used it may be impossible to determine the index of highly opsonic immune sera because the number of bacilli taken up under their influence may be so great that it is impossible to count them.

When a recently isolated paratyphoid organism is used, dilution is not necessary since this organism, as a rule, grows much less luxuriantly than the typhoid bacillus, and spontaneous phagocytosis is either slight or entirely absent. Old cultures, however, may show spontaneous phagocytosis.

*Serum.*—Blood is collected from the ear into a small U-tube which is centrifuged; the supernatant serum is employed in the experiment. The normal control serum consists of a mixture in equal parts of sera derived from three or more persons.

Because of the marked lytic effect of both normal and typhoid sera, it has been our practice to heat the sera to 56° C. for 20–30 minutes in order to destroy the typhoid complement and thus be able to compare the effect of the thermostable opsonic substances in different sera. We have found that normal human and normal rabbit serum always retain a distinct opsonic effect upon typhoid and paratyphoid bacilli after inactivation at 56° C.

*Mixture of elements.*—Equal parts of washed leucocytes, heated serum, and bacillary suspension are thoroughly mixed in a capillary pipette, and the mixture incubated at 37° C. for 30 minutes.

*Estimating the index.*—Smears are made in the usual way, stained with carbol-thionin and the number of organisms in from 40 to 100 leucocytes counted. The average number of bacilli taken up by a given number of leucocytes in typhoid or paratyphoid serum is divided by the average number of bacilli taken by as many leucocytes in the normal serum and the quotient is the opsonic index. Inasmuch as the patient's serum as well as the normal control serum are both heated, it is consequently the thermostable opsonins in both that are determined and compared quantitatively.

#### DETERMINATIONS OF OPSONIC INDEX IN TYPHOID FEVER.

We have found that there is a rather wide variation in the number of bacilli taken up in various normal sera even though the same technic be followed rigidly. Furthermore, the serum of an individual may be

highly opsonic for the same organism one day and very moderately so the next, as shown by the following results obtained on two days with sera from four normal individuals:

	May 6	May 10
1.....	54	58
2.....	47	100
3.....	24	246
4.....	186	62

It is also a striking fact that various strains of typhoid bacilli give different indices with any given typhoid serum. This fact was also observed by Klien. So marked is this difference that we cannot define any given course as the one representing the typic opsonic variation in typhoid fever. We have even met with one strain that gave a persistently low index as compared with other strains. The general course may, however, be the same with many strains. Charts

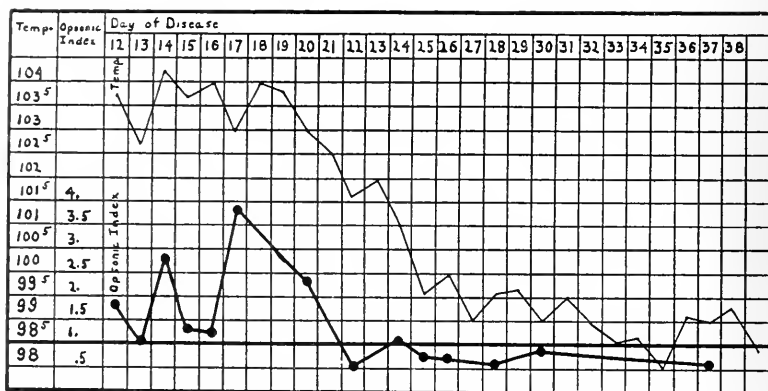


CHART 1.—Typho-opsonic index for bacillus from patient's blood in typhoid fever.

1 and 3 show the index obtained for the bacillus from the patient's blood in a case of typhoid fever. Further observations are desirable.

We have frequently noted that a recently isolated organism will be taken up readily in immune serum, but only to a slight extent in normal serum. After a variable number of transplantations on various media the organism may become readily susceptible of phagocytosis in both normal and immune sera. As an example of this are given the figures obtained on different occasions with a bacillus isolated from the blood of a typhoid-fever patient: On April 23 the

patient's serum gave an average phagocytosis of 1.25, while the normal gave only 0.06; on April 25 the immune gave 6.6, the normal 1.85; on April 30 the immune gave 1.45 and the normal 0.93. Here phagocytosis was marked in the immune serum from the beginning, but was at first very weak in normal serum. In this and other similar experiments very special care was taken in order to secure suspensions of the same density.

The indices obtained for staphylococcus, streptococcus, diphtheria bacillus, pneumococcus, and tubercle bacillus with immune serum rich in opsonin for typhoid bacilli are not correspondingly high, but are as a rule normal. There is usually obtained, however, a high index with paratyphoid bacillus. In fact it has been our experience that the opsonins arising in the course of typhoid fever are generally if not constantly equally capable of stimulating phagocytosis of the paratyphoid bacillus.

We have found, too, that while the index obtained in typhoid fever with the typhoid bacillus may not be high at a given time, the index obtained with the paratyphoid bacillus at the same time is often very high. This high index may be obtained with several paratyphoid strains. Thus in 12 examinations of blood from various typhoid cases in which the indices were obtained at the same time with three strains of paratyphoid, and four of typhoid bacilli, the four organisms giving the highest average indices were three paratyphoid and one typhoid strain.

In the cases in which we examined the serum with one paratyphoid strain the index was found uniformly high early in the disease, approaching normal as convalescence set in, often rising again during the first part of convalescence, again to approach normal during its close. In one case a relapse occurred and there was a repetition of the characteristic curve (see Chart 2).

As stated, various strains of typhoid and paratyphoid bacilli may give different indices with the same serum. Although the rise of the index with one strain is not necessarily followed by a rise in the index with other strains, yet one often sees a corresponding rise and this rise may be quite accurately proportional as shown in Table 1. Here it is seen that during the course of a month, in a typhoid patient, the relation of the index with the paratyphoid bacillus "P" to that obtained with

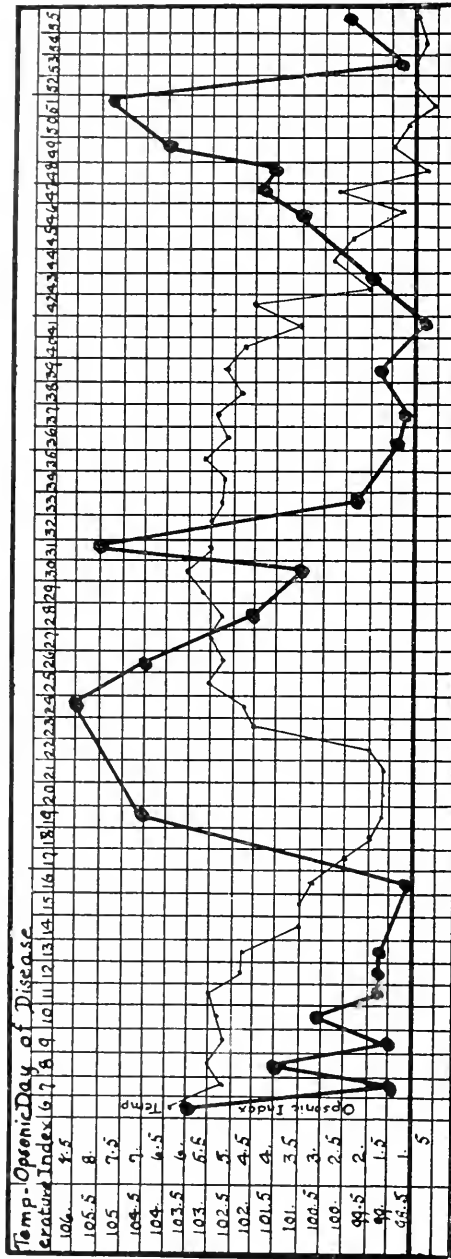


CHART 2.—The opsonic index in typhoid fever for paratyphoid bacillus.

TABLE I

DATE	BACILLUS	INDEX	—PROPORTION—		
			P	M	G
Jan. 6.....	P	7.00			
	M	0.60	10	1	
" 10.....	P	5.88			
	M	0.51			
	G	1.74	11	5	3
" 12.....	P	3.11			
	M	0.20			
	G	0.96	10	7	3
" 20.....	M	0.21			
	G	0.63			
" 24.....	P	3.4			
	G	1.11			
Feb. 10.....	P	0.9			
	M	0.75			

P=Paratyphoid

M=Typhoid

G=Typhoid

the typhoid bacillus "M" was approximately 10 to 1; this, regardless of the fact that one was a high index, the other uniformly subnormal. The relation of the index of bacillus "G" to that of "P" and "M" was 3 to 1. This relationship is not infrequently observed.

In a case of typhoid fever several examinations were made by the dilution method used by Klien at the same time as the index was determined in the usual way (Chart 3). The patient's own organism was used; the results obtained by the two methods in this case agree very well. We regret that more observations of this kind have been impossible up to the present time. It is strongly suggested

by the results thus far obtained, however, that the general outlines of the opsonic curves obtained by the two methods will be the same, assuming, of course, that heated serum be used and an organism that does not give spontaneous phagocytosis.

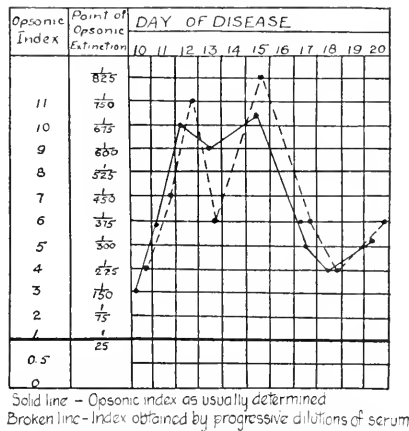


CHART 3.—Comparison of opsonic index for bacillus from patient's blood in typhoid fever as usually determined and as obtained by progressive dilutions of the serum.

In most of the cases studied the diagnosis of typhoid fever was established by the isolation of the typhoid bacillus from the patient's blood.

#### THE FORMATION OF SPECIFIC OPSONINS AFTER INJECTION OF RABBITS WITH KILLED TYPHOID AND PARATYPHOID BACILLI.

A study of the opsonins in rabbits following inoculations of dead cultures of the typhoid and paratyphoid bacilli brought forth certain definite conclusions. Preliminary to the experiments the indices of

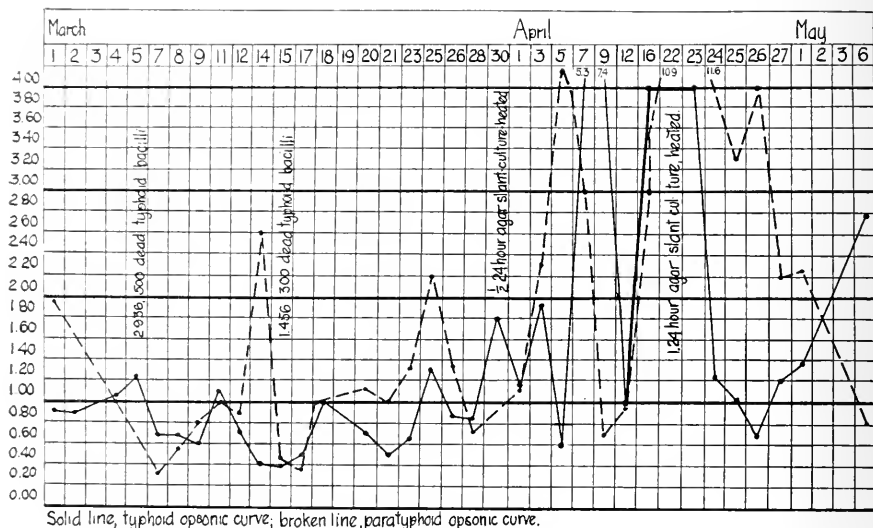


CHART 4.—Opsonic indices for typhoid and paratyphoid bacilli in rabbit injected with killed typhoid bacilli.

three normal rabbits were estimated on five successive days in the usual manner, so as to obtain some idea of the normal variations. For the typhoid bacillus the normal maximum and minimum appear to be 1.3 and 0.7 respectively. In the estimation of the index with the paratyphoid bacillus a greater variation was found, since normal rabbit serum appears to possess only a moderate amount of opsonin for this organism only a slight increase or decrease in the paratyphoid-opsonic content of a single serum to be tested may produce quite considerable differences in the opsonic index from day to day. For this organism the maximum index was 1.97; the minimum, 0.7; and the



average of the five estimates, 1.32. There was not any close correspondence between the typhoid and paratyphoid opsonic index in the same rabbit on the same day. For example in rabbit A the typhoid opsonic index was 0.96; the paratyphoid index at the same time was 1.97.

The observations on the injected animals extended over a period of 67 days. In the beginning the opsonic index of each rabbit to both the typhoid and paratyphoid bacillus was taken daily; later at less frequent intervals. The serum of three normal rabbits, collected at

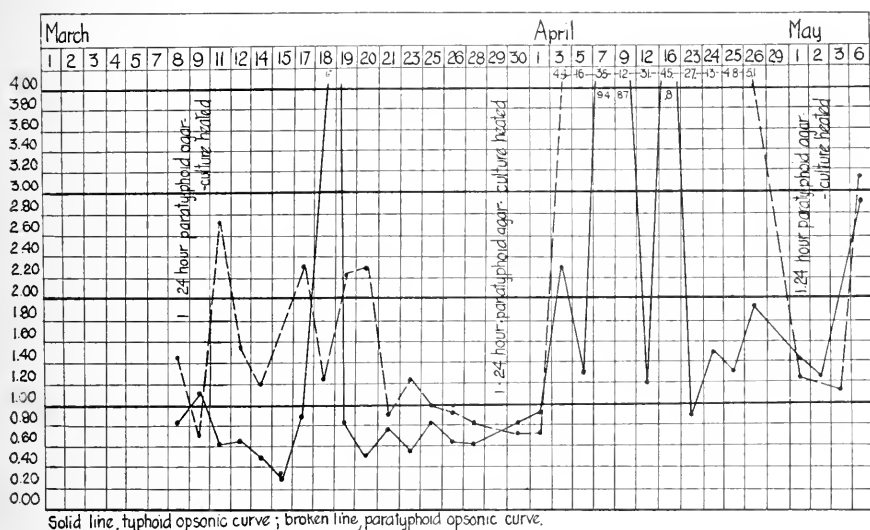


CHART 5.—Opsonic indices for typhoid and paratyphoid bacilli in rabbit injected with killed paratyphoid bacilli.

the same time as the immune serum, was used as the normal standard. The serum was heated for 15 to 20 minutes at 56 to 58° C. Human leucocytes were used as phagocytes. Otherwise the Wright technic was followed.

Subcutaneous injections of suspensions of killed bacteria from 24-hour agar slant growths of one typhoid and two paratyphoid strains were given at intervals of 10 days or more. Rabbit A received one 24-hour agar slant growth of *B. typhosus* (strain 8) suspended in sodium-chloride solution and heated to 66° for two hours. Rabbit B received a 24-hour agar slant growth of both *B. typhosus* (strain 8)



place immune rabbit sera treated with large quantities of typhoid bacilli show greater diminution in the amount of typho-opsonin than of paratypho-opsonin and vice versa. In these absorption experiments there was used in each case the same amount of serum and of bacillary suspension or of salt solution (control); after an exposure for 60 minutes to 37° C. all the mixtures were filtered, including those of serum and salt solution, and the ordinary phagocytic experiment was now carried out by mixing equal parts of serum, leucocytic suspension, and bacillary suspension. The actual results are shown in

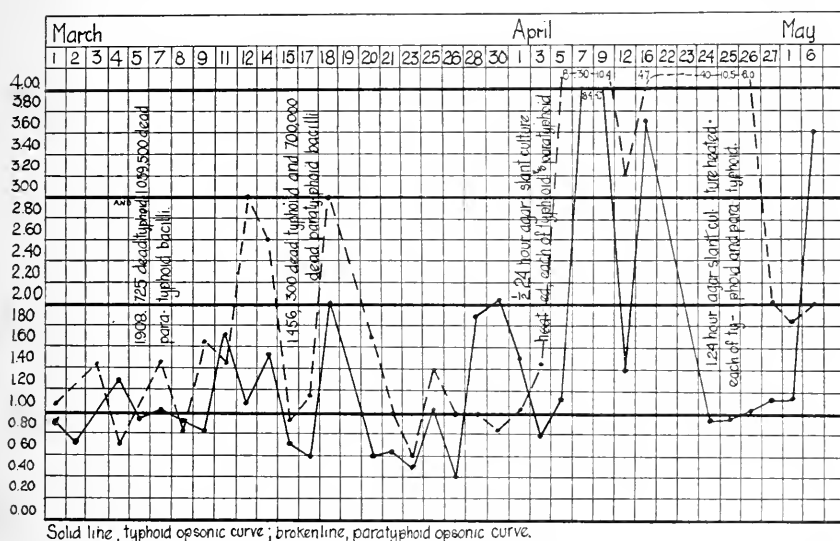


CHART 7.—Opsonic indices for typhoid and paratyphoid bacilli in rabbit injected with killed typhoid and killed paratyphoid bacilli.

Table 2, and they plainly indicate that the serum contains two distinct opsonic bodies. It must be noted that the paratyphoid opsonin produced after injection with typhoid bacilli is partially absorbed by typhoid bacilli; also that the typhoid opsonin produced by inoculation with *B. paratyphosus* is partially absorbed by *B. paratyphosus*.

Contrary to the results obtained with dead streptococci, there appears to be a gradual increase in the rise of the opsonic index after each successive injection. Thus the first injection of rabbit A was followed by a marked negative and almost no positive phase; whereas after the second and third injections the negative phase became less

TABLE 2  
THE EFFECT OF TREATMENT OF ANTITYPHOID AND OF ANTIPARATYPHOID RABBIT SERA WITH TYPHOID  
AND PARATYPHOID BACILLI

SERUM	AVERAGE NO. OF BACILLI PER LEUCOCYTE	
	Typhoid Bacilli	Paratyphoid Bacilli
Antityphoid Serum untreated	7.8	1.0
" " treated with Typhoid Bacilli	1.0	0.5
" " " " Paratyphoid Bacilli	7.4	0.36
Antiparatyphoid Serum untreated	3.0	2.0
" " treated with Typhoid Bacilli	2.0	2.0
" " " " with Paratyphoid Bacilli	1.0	0.016
Nace Solution only	0.016	0.015

marked and the positive much greater. The enormous indices obtained after the third injection are interesting, especially when we note that they remained high for a considerable length of time.

The opsonins that develop after injections of typhoid and paratyphoid bacilli do not affect the index to the tubercle bacillus; and usually a given vaccine produces a greater rise in the opsonic index toward the organism injected than to the other of the organisms used in these experiments. The virulence of the typhoid strain seems not only to affect the amount of phagocytosis but curiously enough also the opsonic index obtained. Strain 8' in the table is a two-month-old agar culture of strain 8; strain 8 is the parent organism used in these experiments and has been transferred to fresh broth daily; strain 8'' is strain 8 modified by being passed through a series of 12 guinea-pigs, the last pig being killed by  $\frac{1}{4}$  c.c. of a broth culture injected intraperitoneally. The experiment was done with the same serum, on the same day, under exactly the same conditions throughout and with suspensions of the organisms which appeared of exactly the same

TABLE 3  
THE EFFECT OF VIRULENCE OF THE STRAIN UPON THE OPSONIC INDEX.

STRAIN	SERUM OF RABBIT A		SERUM OF RABBIT B		SERUM OF RABBIT C		SERUM OF RABBIT VI	
	No. of Bacilli in 50 Leucoc.	Index	No. of Bacilli in 50 Leucoc.	Index	No. of Bacilli in 50 Leucoc.	Index	No. of Bacilli in 50 Leucoc.	Index
8'	65	1.02	78	1.21	72	1.13	63	0.98
8	..	0.83	..	1.94	..	1.29	..	0.62
8''	17	0.46	23	0.62	32	0.86	23	0.62

density. It is seen that the opsonic index obtained with the virulent organism is lower in each serum than that with the less virulent strains. At present we offer no explanation of this phenomenon.

#### SUMMARY.

After heating normal human and rabbit serum to 56-58° C., there persists in these sera in appreciable degree the power to promote phagocytosis of typhoid and paratyphoid bacilli by washed human leucocytes. As the result of spontaneous infection as well as artificial inoculation with these bacilli this power of the serum may undergo profound modifications and may be augmented in very high degrees. The exact determination of this opsonic power with respect to typhoid bacilli in the course of typhoid fever was found to present many difficulties on account of inconstancy and variations in the induced and spontaneous phagocytability of the typhoid bacillus, demanding much further study, whereas with respect to paratyphoid bacilli the variations of the opsonic power of the serum of typhoid-fever patients are more easily determined and appear to follow a fairly typical course, the opsonic index as determined by comparison of heated normal and typhoid sera, being high early in the disease when the patients ordinarily come under observation, approaching normal more or less gradually as convalescence sets in. In rabbits injected with either dead typhoid or dead paratyphoid bacilli there develop in each case immune opsonic substances for both these organisms; these substances, however, appear to be largely specific, no matter whether produced in response to the injection of the one or the other of the bacteria.

## THE OPSONIC INDEX IN DIPHTHERIA.\*

RUTH TUNNICLIFF.

(From the Memorial Institute for Infectious Diseases, Chicago.)

ACCORDING to Wright<sup>1</sup> diphtheria bacilli are insensible to the opsonic action of blood fluids. This conclusion is based upon experiments showing that there is as much (or, in one case, more) phagocytosis in the presence of normal human serum heated at 60° C. as in unheated. In order to study this question four strains of typical diphtheria bacilli furnished me by Dr. Hamilton were tested. With all of these strains there was no opsonin demonstrable in normal human serum after heating the serum 30 minutes at 50° C. This was found to be true also of serum from diphtheria patients when tested with strain "N," the organism employed in the subsequent experiments.

Reque<sup>2</sup> also found that when washed leucocytes are mixed with normal human or dog serum heated to 58-60° for 15-30 minutes, there is practically no phagocytosis with diphtheria bacilli. It is possible that the strain which Wright used was subject to the spontaneous phagocytosis which has been observed to occur with occasional strains of diphtheria bacilli.

As the course of the specific opsonic index in certain acute infections has been found to possess characteristic features that harmonize well with the general clinical picture, rising above normal as the symptoms decline, it was thought it would be of interest to determine if this were also true in regard to diphtheria. Both the diphtheria- and the streptococco-opsonic indices were estimated on account of the close association of streptococci with tonsilar infections.

The Wright method of estimating the opsonic index has been employed for the most part. The suspensions are made from 24-, occasionally 48-hour, cultures on plain agar or Löffler's blood serum. The fluid of condensation is discarded on account of its containing clumps. A suspension is made in enough 0.85 per cent salt solution

\* Received for publication December 20, 1907.

<sup>1</sup> *Proc. Royal Soc.*, 1904, 73, p. 128.

<sup>2</sup> *Jour. Infect. Dis.*, 1906, 3, p. 441.



to give an average count of 1.5 diphtheria bacilli (the suspension containing about 300,000 bacilli per c.mm.) and 3-5 streptococci per leucocyte in the control specimen with normal serum. The Wright method of determining the opsonic index has been criticized on account of inaccuracy due to variation in the thickness of the bacterial suspensions. Suspensions of diphtheria bacilli varying from 125,000 to 1,000,000 bacilli per c.mm. were found, however, to give practically the same indices, as seen from the table:

TABLE I

Suspension (No. bacilli per c.mm.)	Opsonic Index
1,000,000.....	1.16
750,000.....	1.16
600,000.....	1.2
500,000.....	1.15
450,000.....	1.2
425,000.....	1.5
375,000.....	1.2
300,000.....	1.3
250,000.....	1.2
250,000.....	1.2
150,000.....	1.3
125,000.....	1.2

The serum from three normal individuals is pooled and used as the normal control. The pool and the patient's serum are collected within an hour of each other and removed from the clot at nearly the same time because of the difference in the quantity of opsonin in serum removed at different times after clotting. The washed blood (leucocytes) is obtained from an individual whose red blood cells are not susceptible to isoagglutination. Fifty polymorphonuclear neutrophils are counted, 25 at each end of the slide. If these two counts do not closely correspond, more leucocytes are counted. Clumps of bacteria as well as clumps of leucocytes are discarded. Isolated or paired leucocytes only are counted.

In my preliminary tests I found that the diphtheria index in normal adult persons may vary between 0.92 and 1.1, except that in one case it was 0.8 and in another 1.2. The normal streptococcal index I found to vary from 0.9 to 1.1.

As all of the patients examined received diphtheria antitoxin, it seemed advisable to determine if this injection had any immediate influence upon the opsonic index for diphtheria bacilli. A normal man was injected with 12,000 units and his index taken several days

before and after the injection, but at no time was it found outside the normal limit, varying from 0.92-1.1.

The diphtheria- and streptococco-opsonic indices of 14 diphtheria

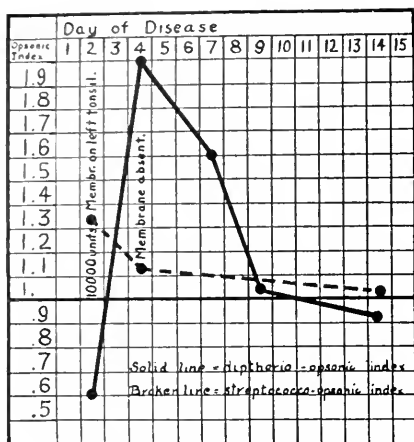


CHART 1.—Opsonic indices for *B. diphtheriae* and streptococcus in diphtheria (man, 30 years, no fever).

patients have been observed as a rule daily throughout the course of the disease. The diagnosis of diphtheria was confirmed in each case by bacteriological examination, except in two laryngeal cases in which the cultures were negative. The cases occurred at the Cook County Hospital, in the services of Dr. W. L. Baum and Dr. Geo. H. Weaver, to whom I am indebted for the privilege of making the examinations.

In eight cases, all examined early in the disease, the diphtheria index was found at first to be below normal, ranging between 0.4 and 0.7. Of the six cases in

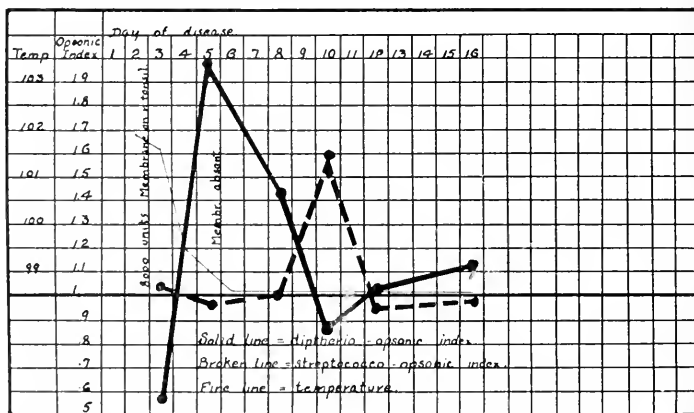


CHART 2.—Opsonic indices for *B. diphtheriae* and streptococcus in diphtheria (man, 36 years).

which no negative phase was observed, two were first examined after the membrane had disappeared and three were laryngeal cases.

In all of the patients the disappearance of the membrane and improvement in the symptoms were accompanied with a rise above normal of the indices which varied from 1.3 to 2.2, the average

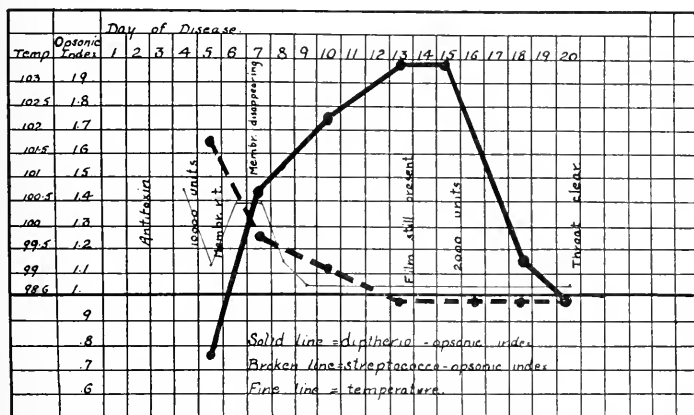


CHART 3.—Opsonic indices for *B. diphtheriae* and streptococcus in diphtheria (girl, 10 years).

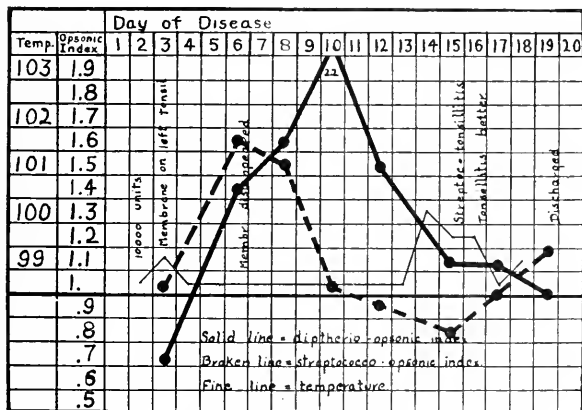
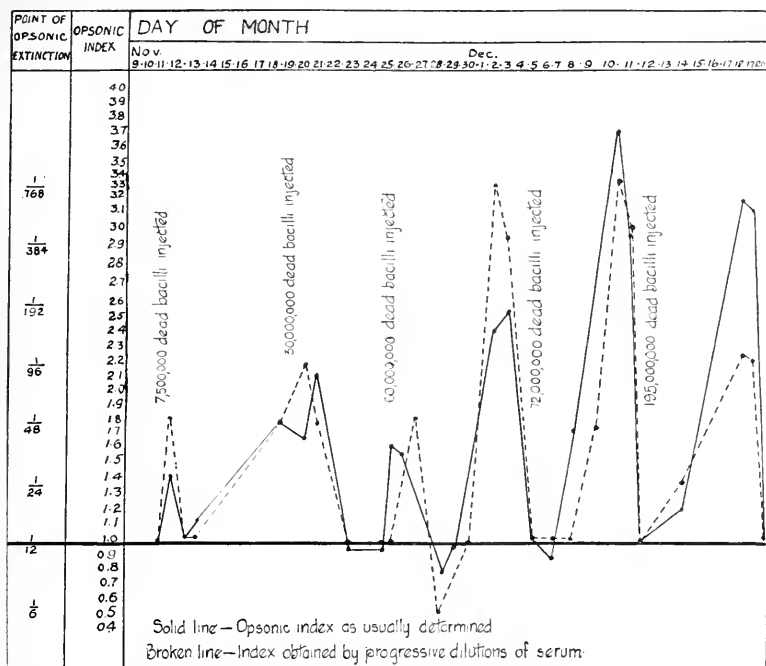


CHART 4.—Opsonic indices for *B. diphtheriae* and streptococcus in diphtheria (woman, 25 years).

highest point being 1.9. This rise was followed by a fall to normal in from 2–9 days (see Charts 1, 2, 3, 4).

In two cases the streptococcal index remained normal. The index was found below normal only twice. In the majority of cases the rise in this index occurred earlier in the attack than that of the





days and an increase during convalescence. Although in the majority of my cases the low and high indices occurred earlier than his, our observations on the whole agree.

Rabbits were injected with dead diphtheria bacilli to determine whether or not the opsonic indices would correspond to the course of the index in cases of diphtheria.

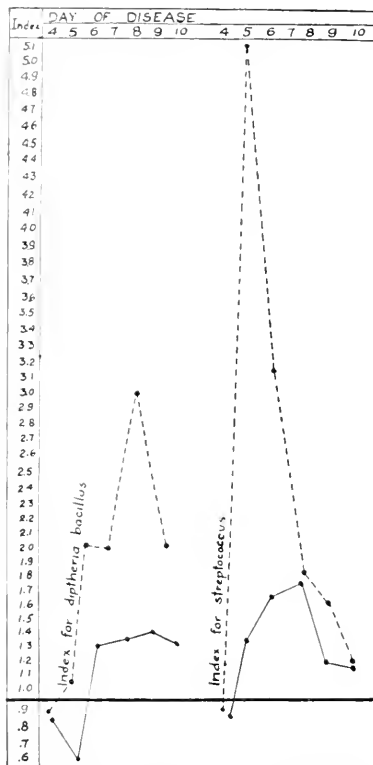


CHART 9.—Opsonic indices for *B. diphtheriae* and streptococcus in diphtheria (woman, 30 years) as obtained after incubation of mixtures at 37° C. for five minutes (solid line) and after incubation at 45° C. for five minutes (broken line).

on account of the rapid destruction of the leucocytes at 45° C.

Klien<sup>1</sup> observes that the opsonic index in rabbits injected with typhoid bacilli, as estimated by the Wright method, does not show the real amount of opsonin present. He therefore diluted the normal and

The first injection of 17,000,000 dead bacilli produced a prolonged negative phase, followed by only a slight rise on one day, the 9th day after injection (Chart 6). Charts 7 and 8 show the effect of repeated injections of a smaller number of organisms. Each injection was followed by a rise in the index. In only the one case was a negative phase demonstrated, namely after injection of 72,000,000 organisms, showing apparently that large doses only are likely to produce negative phases in rabbits.

In five cases of diphtheria, the mixtures of serum, leucocytes, and suspension were incubated at 45° C. for five minutes. Chart 9 shows that the indices differ in height, when incubated at 37° and 45° C.; otherwise they correspond fairly closely in their course. Five minutes was chosen for the time of incubation

<sup>1</sup> Bull. Johns Hopkins Hosp., 1907, 18, p. 245.

immune serum with normal salt solution to the point where phagocytosis recognizably exceeded spontaneous phagocytosis and in this way measured the opsonic power. It was thought well to employ this method in connection with the present investigation. As there was a slight spontaneous phagocytosis with the diphtheria bacillus employed, a control test with salt solution was always made. I found that with a suspension of about 300,000 bacilli per c.mm. normal opsonin in

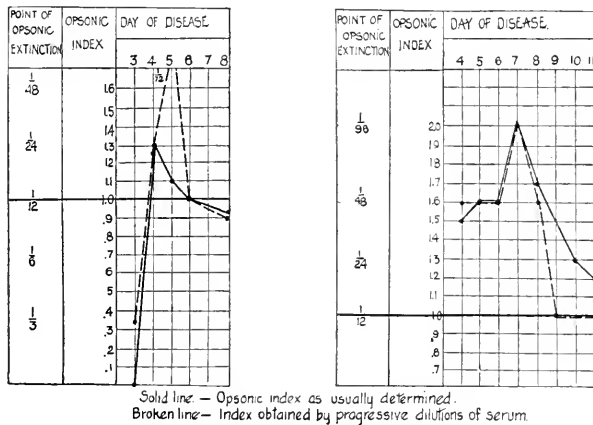


CHART 10.—Comparison of opsonic index for *B. diphtheriae* as usually determined and index obtained by progressive dilutions of the serum in a mild, uncomplicated case of diphtheria.

human and rabbit serum disappeared at a dilution of 1-24, there always being some present at 1-12. The percentage and the phagocytic indices were found to closely agree, calculating the percentage index taking much less time. The opsonic indices of two diphtheria patients and two rabbits injected with dead diphtheria bacilli were estimated in the usual way, at the same time as the opsonic power was calculated by diluting the serum to the point just exceeding spontaneous phagocytosis. As seen in Charts 7 and 8, the two methods give in this case results which correspond very closely.

Dean<sup>1</sup> found that there was no direct proportion between the serum concentration and the number of organisms ingested, there being as many or more with the half, quarter, or even one-eighth concentration as with the full strength. He tested staphylococci and tubercle bacilli with serum of several species of animals. In 85 experiments

<sup>1</sup> *Proc. Royal Soc.*, 1907, B. 79, p. 399.

with the diphtheria bacillus, using both human and rabbit serum, only once did I find as many organisms taken up in the half-strength as in the full.

#### CONCLUSIONS.

The diphtheria bacillus is sensible to the opsonic action of human and rabbit blood serum.

In diphtheria the opsonic index for diphtheria bacilli is generally below normal at the onset of the disease. As the membrane disappears and the symptoms subside the index rises considerably, returning to normal in from two to nine days.

In the majority of cases there is a rise in the streptococco-opsonic index in diphtheria. The indices for diphtheria bacilli and streptococci rarely correspond.

The injection of dead diphtheria bacilli in suitable number into rabbits is followed by a rise in the index.

The indices obtained after incubating the mixtures at  $45^{\circ}$  C. are higher but correspond fairly closely with the course of the indices obtained after incubation of the mixtures at  $37^{\circ}$  C.

By diluting the normal and the immune serum until phagocytosis practically ceases, the comparative opsonic power of the sera on diphtheria bacilli can be measured. The curves representing the opsonic power determined in this way correspond almost exactly with the curves of the opsonic indices estimated by Wright's method.

The suggestion may be permitted that the injection of dead diphtheria bacilli may prove of some service in ridding the throat of bacilli in the case of chronic carriers and convalescents, as the experiments show that in rabbits such injections are harmless and at the same time cause decided increase in the opsonic power of the blood upon diphtheria bacilli.



# SPLENOMEGALY AND BANTI'S DISEASE.\*

## WITH REPORT OF A CASE

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### INTRODUCTION.

The following case of so-called "Banti's disease" is reported for two reasons: first, because full clinical histories of such cases with detailed description of the results of anatomical examination are not numerous in the literature; second, and chiefly, because the spleen in this instance presents a very peculiar and rare form of disseminated thrombosis of the splenic sinuses and the small veins draining them, with deposition of iron-containing pigment in the thrombosed regions. Descriptions of only three similar spleens could be found in the literature, the search in which was limited chiefly to that relating to Banti's disease. The three cases referred to are those of Hamill,<sup>1</sup> Borissowa,<sup>2</sup> and Stengel;<sup>3</sup> but these authors only mentioned the condition without describing it fully or offering any explanation for it.

### REPORT OF CASE.

The clinical history of this case is given through the courtesy of Dr. F. Billings, and the anatomical report through the kindness of Dr. A. D. Bevan.

The patient was a fireman at a stationary engine, and was 33 years old when he first came under observation, October 24, 1900. The family history was negative. He had the usual diseases of childhood, but otherwise was always healthy, until the beginning of the present trouble. Eighteen months previously, hemorrhage from the stomach occurred, the amount of which was unknown and there had been no recurrences. On October 10, 1900, the patient was seized with severe pains in the left hypochondrium, while stooping over shoveling coal. He was slightly nauseated at the time but did not stop work. Since then he had pain in the same region on going to work after a full meal; but no pain if he remained quiet. He had to stop work October 17 because of the pain.

\* Received for publication November 20, 1907.

<sup>1</sup> Hamill, *Archives for Pediatrics*, 1902, 19, p. 641.

<sup>2</sup> Borissowa, *Virchow's Arch.*, 172, p. 108.

<sup>3</sup> Stengel, *Am. Jour. Med. Sci.*, 1904, 128, p. 407.

Examination at the time of entrance in the Presbyterian Hospital of Chicago showed the chest organs normal. The spleen was palpable one inch below the costal margin; felt tense, smooth, and firm, and moved with respiration. A few enlarged glands were palpable in the neck. There were numerous leucodermic patches over the skin, which began one year previously. The urine was normal. Blood examination showed: 4,000,000 reds; hemoglobin, 55 per cent; color index, 0.68; leucocytes, 4,800.

The patient was under observation at intervals for a period of four years. During this time the blood remained practically the same. The reds were constantly below normal, once falling as low as 2,584,000; the color index was less than 1.00 except at one estimate. The leucocytes ranged usually between 3,000 and 4,000, once being as low as 1,700. Differential leucocyte counts showed nothing particularly abnormal; once nucleated reds were found, and three times a few myelocytes.

The spleen steadily enlarged. There was some loss of strength. Gastric hemorrhages grew increasingly severe and more frequent. In July, 1904, the patient suffered an attack of diphtheria; recovered, and felt unusually well for some weeks. The increasing severity of the gastric hemorrhages induced the patient to submit to splenectomy, October 11, 1904. Death occurred from hemorrhage, due to rupture of a dilated vein in an adhesion anterior to left kidney.

Post-mortem examination showed the following: General anemia; localized sub-diaphragmatic fibrous peritonitis with torn adhesions and calcareous areas; intrahepatic cholelithiasis and fibrous pericholangitis; phleboliths in the pancreas; patches of red marrow in femur.

The esophagus was smooth. The rugae of the stomach were prominent and did not obliterate on stretching. Many minute hemorrhagic points and small vessels were seen upon the surface of the gastric mucosa, but no dilated veins were present. Other organs showed nothing unusual, except the spleen.

Microscopically the liver showed an increased amount of periportal fibrous tissue and slight fatty change.

The spleen, after preservation in museum fluids, weighed more than 1,000 grms., and measured  $25.5 \times 13.5 \times 7$  cm. The general shape is well preserved. The external surface shows a few tags of adhesions and two calcareous areas in the capsule, which do not extend into the spleen tissue. The vessels at the hilum are cut off very close and no abnormality of these vessels is mentioned in the autopsy protocol, but the portion left attached shows the same evidence of sclerosis.

Disseminated over the cut surface are numerous round, oval, or branched reddish-brown areas about 2 mm. in diameter (Fig. 1). These areas give an intense reaction for iron with potassium ferrocyanide and hydrochloric acid. Careful dissection of these from the surrounding spleen pulp shows a plump fusiform mass about the size and shape of a grain of wheat. Two or more of these may unite by their proximal ends and, by a common stem, become attached to a small patent artery, or they may become attached to the artery singly.

Microscopic examination shows practically uniform changes throughout the spleen. Many of the Malpighian bodies appear normal. In others there has been an ingrowth of connective tissue, either from the periphery, or along the central artery, or from both sources. A few have undergone hyaline degeneration.

The capsule, the trabeculae, and the reticulum show marked proliferation of connective tissue, much of which is of a distinctly embryonal type. In the inner zone of

the capsule and the lateral zones of the trabeculae, there are, in this connective tissue, numerous wide, round, or oval spaces lined by a single layer of endothelium and completely filled with closely packed red blood cells.

Proliferation of endothelium, if present at all, is extremely slight. There is nothing even suggestive of the conditions described by Gaucher<sup>1</sup> and Bovaird.<sup>2</sup>

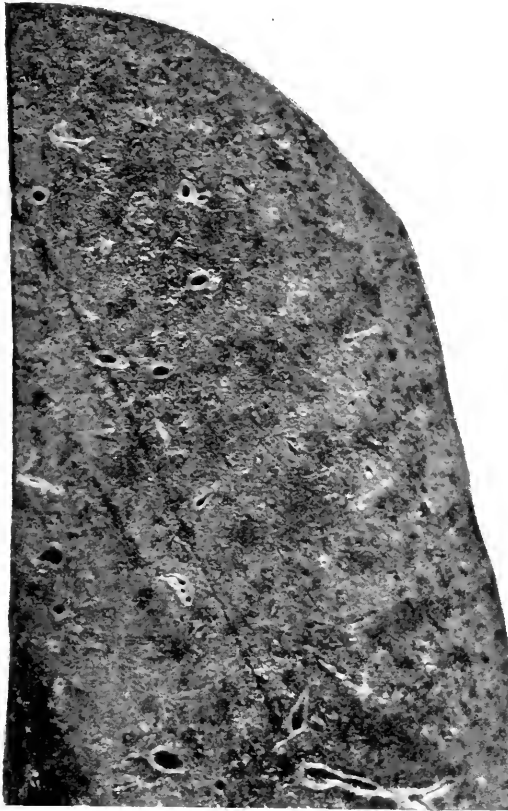


FIG. 1.—Cut surface of spleen showing areas of deposit of pigment.

An occasional multinuclear cell, similar to those found in bone marrow, is seen.

In the spleen pulp are numerous areas, some quite large, composed of densely crowded, sometimes more or less altered, red blood corpuscles, among which is a fine meshwork composed of fusiform cells. This network suggests the appearance of pulp spaces enormously dilated by the contained blood. Within these regions are often seen wide blood vessels, quite full of blood, and having extremely thin walls

<sup>1</sup> *Semaine med.*, 1892, 12, p. 331; Abstract in *Schmidt's Jahrb.*, 1892, 236, p. 137

<sup>2</sup> *Am. Jour. Med. Sci.*, 1900, 120, p. 377.

which in places appear to have ruptured. These vessels are too wide to represent distended sinuses, but they may represent dilated pulp veins of Weidenreich.<sup>1</sup>

In most of the sections there are seen accumulations of pigment which represent sections of the fusiform masses described above (Fig. 2). With hemotoxylin and eosin this pigment is yellowish brown or purple, both colors occurring in the same area; it gives a beautiful iron reaction. The deposit has occurred chiefly in coarse fibers which are often fragmented. The tissue within these regions is often more or less hyaline, and the regions are quite definitely marked off. Each contains a blood vessel,

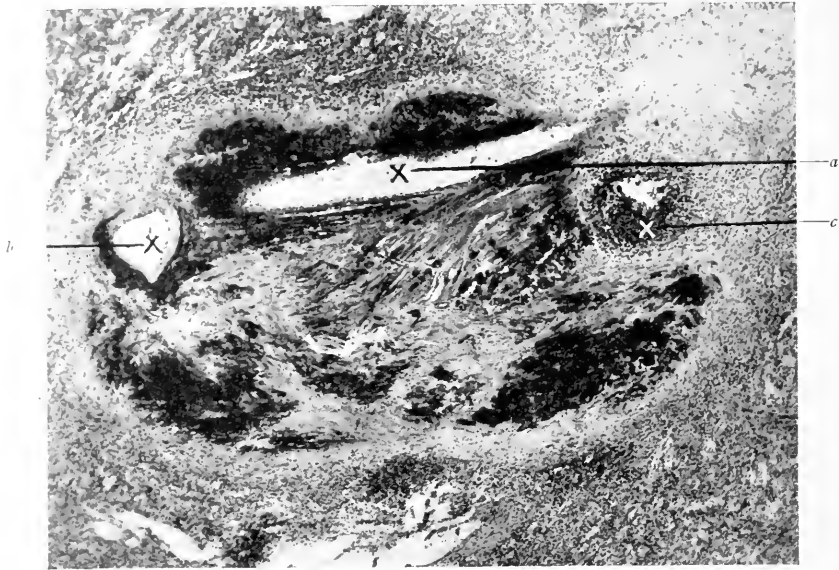


FIG. 2.—Section showing deposit of pigment in fibrous tissue and vessel wall in the spleen: *a* artery cut obliquely; *b*, artery cut transversely; *c*, Malpighian body.

a part of whose wall is sometimes pigmented. The size of the pigmented region varies roughly with the size of this vessel.

Ehrlich<sup>2</sup> found that iron-containing elastic and collagen fibers of scars in the spleen were markedly degenerated, and, further, that the elastic fibers in the neighborhood of hemorrhages become impregnated with iron-containing compounds derived from the blood pigment and that such fibers can afterward take up calcium. The deep purple stain with hemotoxylin indicates that many of the fibers in the pigmented areas of these sections have also taken up calcium.

About 300 sections parallel to the capsule were made, and every fourth one stained and mounted in order, thus making a series of 75 or 80 sections of these masses of pigment in cross-section throughout their entire length.

<sup>1</sup> *Arch. f. mik. Anat.*, 1921, 58, p. 247.

<sup>2</sup> *Centralbl. f. allg. Path. u. path. Anat.*, 1906, 17, p. 177.

In none of these sections does the artery of a pigment mass show an occluding thrombus. It is always patent, though in some instances its walls are somewhat collapsed. The artery, distal to the deposit, is surrounded by a narrow zone of connective tissue, and is probably a small "trabecular artery" (Weidenreich). In approaching one of these deposits from the capsule one first notices red blood cells in the tissues surrounding the artery. Nearer the deposit these red cells become more and more numerous and the tissue elements are pushed wide apart forming a wide-meshed network of coarse and fine fibers among the red cells, which sometimes coalesce into a homogeneous mass. This condition extends beyond the zone of connective tissue mentioned, into the pulp. It is in the finer of these fibers that the first pigment is seen; in the very next section, however, the coarse, fragmented fibers also contain pigment. A zone of red blood cells surrounds the whole mass throughout its whole length. Two of these pigmented regions in different parts of a section can frequently be seen to unite in a more proximal section with junction of their arteries. In sections immediately proximal to the deposit one finds an accumulation of red cells similar to that at the distal end. The tissues in this location are greatly distorted, but in each of these regions there is an area, a short distance from the artery, which represents an enormously dilated, thin-walled vein.

The following is offered as a possible explanation for the deposition of these calcium- and iron-containing pigments. Since they immediately surround the artery, and since serial sections show the veins in close relation to this vessel proximal to the pigmented region, it is believed that the deposit of pigment occurred in thrombi of such veins, which thrombi extended distally and involved sinuses drained by them.

The accumulation of red blood corpuscles and the distension of the tissue spaces and the spleen sinuses as described could have been produced by some obstruction to the outflow of blood from the territory so involved. No definite cause for such obstruction could be found in the sections, however. The moderate fibrotic changes in the liver certainly offered some obstruction to free outflow from the entire spleen. It might be suggested, furthermore, that in such a huge spleen, the total blood space must have been enormously greater than normal. This being true, unless the afferent and efferent vessels were dilated in proportion to this increase of blood space, the rate of flow of blood through the spleen would, of necessity, be diminished. This slowing of the current of blood, together with any change in the walls of the smaller veins, such as slight localized endophlebitis, could easily lead to the formation of a thrombus, which, extending into the radicles and sinuses emptied by the affected vein, would produce the conditions described. Given this region of thrombosed vein and sinuses, a

proper soil is at hand for the deposition of both iron- and calcium-containing pigment.

This is merely suggested as an explanation of the presence of the masses of pigment, since in the sections nothing was found to account for their origin definitely.

#### GROUPING OF CASES. I.

A comparison of this case with others found in the literature indicates that they constitute a distinct group with rather definite clinical manifestations and anatomical changes. In addition to the enlarged spleen and the anemia of a chlorotic type, cases of this group are characterized clinically by gastric hemorrhages; frequent and relatively early ascites, often preceded by changes in the size of the liver; sometimes by leukodermic patches on the skin; and very rarely by jaundice. A majority of the patients are over 20 years of age at the onset of the disease. Anatomically, the condition is characterized by hyperplasia of all connective tissue elements of the spleen with, frequently, marked congestion; and less often by cirrhotic changes in the liver, varicose veins in the lower end of the esophagus and the cardia, and increase of red bone marrow. It must be admitted at once, with Simmonds and Umber,<sup>1</sup> that the condition cannot be diagnosed by the anatomical changes alone. Summaries of reported cases follow:

#### SUMMARY OF REPORTED CASES.

*Case 1* (Banti<sup>2</sup>).—Female, age 16. For two years a hard swelling of the spleen. Frequent epistaxis. From time to time irregular attacks of fever. Diarrhea. Ascites, developing without pain or fever. Over heart a murmur propagated to the vessels of the neck. Urine normal. Accessible lymphatic glands not enlarged. Marked anemia of secondary type. Splenectomy. Death from hemorrhage. Autopsy: Stomach, intestines, and kidneys normal. Peritoneum thickened and opaque. Cirrhosis of the liver. The spleen extended to the iliac crest; the capsule and trabeculae were thickened; the glomeruli were larger than normal and the central artery sclerosed; reticulum slightly increased; the veins of the pulp were not dilated.

*Case 2* (Banti<sup>2</sup>).—Female, age 72. Began to experience a sensation of dullness and heaviness in the left hypochondrium, with rapid loss of strength. Some months later noticed a tumor in left flank. Weakness and cachexia increased. Anorexia. Intermittent diarrhea. Grayish color to the skin. No adenopathy perceptible. Liver increased in size. Soft murmur at base of heart and over vessels of neck. Marked

<sup>1</sup> *Munch. med. Wchnschr.*, 1905, 52, p. 772.

<sup>2</sup> *L'Anemia splenica*. Quoted by Bruhl, *Arch. gen. de med.*, 1891, 2, p. 160.

anemia, showing microcytes. Death from pneumonia. Autopsy: Slight ascites, cirrhosis, round cell infiltration and fatty degeneration of liver; spleen weighed 1,255 grms.; capsule and trabeculae were thickened; dilatation of the veins present; sclerosis, often complete, of the Malpighian bodies.

*Case 3* (Coupland<sup>1</sup>).—Female, age 43. Slight continued fever, progressive weakness, and enlarged spleen with attacks of pain, thought during life to be due to hemorrhage into the substance of the spleen and adhesions to the abdominal wall. Anemia not marked and blood, microscopically, appeared almost normal. Autopsy: Spleen reached to the pubis, contained numerous hemorrhages but showed no inflammation of the capsule. Microscopically, there were fibrosis of the trabeculae and atrophy of the Malpighian bodies. The liver was normal.

*Case 4* (Stengel<sup>2</sup>).—Male, age 49. Duration more than two years. Stunted growth. Pigmentation of the skin. Clubbed fingers. Osteo-arthritis. Enlarged spleen. Chloroanemia with leucopenia. Gastric hemorrhages. Diarrhea. Blood-streaked, muco-purulent sputum and evening temperature (no tubercle bacilli mentioned). Early enlargement followed by decrease in size of the liver. Numerous intercurrent infections. Death in stupor. Autopsy: Atrophic cirrhosis of the liver; lobar pneumonia; sclerosis of the aorta; reddish-grey marrow in the femur. Spleen weighed 950 grms., and showed diffuse connective tissue hyperplasia: obliteration of the lumina of many blood vessels; hyaline degeneration of many Malpighian bodies; and numerous deposits of iron-containing pigment "of hemorrhagic origin." "Under high powers, swollen and proliferated endothelial cells were moderately conspicuous in the sinuses but nowhere aggregated to form distinct masses."

*Case 5* (Murrell<sup>3</sup>).—Female, age 31. Duration at least 13 days. Violent hematemeses. Anemia of a secondary type. Leucopenia. Temperature ranging from 99 to 104.4 a few hours before death (temperature thought to have been due to a mastitis). Autopsy: Mucosa of esophagus, stomach, and intestines normal. Spleen weighed nine ounces, and sections showed the connective tissue framework much thickened generally.

*Case 6* (Schlichthorst<sup>4</sup>).—Male, age 22. Duration, four years. Enlargement of the spleen. Severe hematemeses. Autopsy: Marked cirrhosis of the liver; thrombosis of the portal vein; varicose veins in lower end of esophagus with erosion of one of the same. Spleen weighed 1,070 grms. and showed marked thickening of the capsule, trabeculae, and reticulum, and indistinct Malpighian bodies. (From Marchand's very brief summary of this case it could not be distinguished from a case of cirrhosis of the liver, but it was reported with the diagnosis of splenic anemia, or Banti's disease.)

*Case 7* (Osler<sup>5</sup>).—Male, age 35. Recurring attacks of hematemeses and melena for 12 years with excellent health in the intervals. Chronic enlargement of the spleen. No pigmentation of the skin. Blood examination showed nothing abnormal. No leucocytosis. Death during an attack of hematemeses and melena. Autopsy: Chronic hyperplasia of the spleen. Liver showed only fatty change.

*Case 8* (Osler<sup>5</sup>).—Male, age 33. Hematemeses and melena; first attack occurring 10 years previously. Pain in left side. Enlarged spleen. Severe anemia. Leucopenia. Exploratory laparotomy, at which time stomach, duodenum, and liver appeared

<sup>1</sup> *Brit. Med. Jour.*, 1806, 1, p. 1445.

<sup>2</sup> *Am. Jour. Med. Sci.*, 1024, 128, p. 497.

<sup>3</sup> *Lancet*, 1902, 1, p. 1177.

<sup>4</sup> Marburg Dissertation, 1897. Quoted by Marchand. *Munch. med. Wchnschr.*, 1903, 50, p. 403.

<sup>5</sup> *Edin. Med. Jour.*, 1899, 47, p. 441.

normal. Splenectomy and recovery. The spleen showed "a marked degree of fibrous hyperplasia."

*Case 9* (Springthorpe and Stirling<sup>1</sup>).—Female, age 19. Typhoid at seven, jaundice for a few weeks at eight years. Always slightly pale and never quite strong. Anemia with low color index except at one estimation. Marked splenomegaly. Palpitation. Vertigo, frequently quite severe. Hemic bruit. "Splenic pain." Frequent nausea but no vomiting nor hemorrhages. Diarrhea. Splenectomy with complete recovery. The excised spleen weighed 14 $\frac{3}{4}$  ounces. Microscopically it showed "marked increase of fibrillar reticulum of the splenic pulp with diminution in the number of leucocytes;" Malpighian bodies few in number; large amount of coarsely granular pigment; and (apparently) hyaline change in the vessel walls and thickened reticulum.

*Case 10* (Sippy<sup>2</sup>).—Male, age 45. Duration, four years. Splenic swelling. Weakness. Slight nausea and vomiting. Anorexia. Epistaxis. Profound anemia. Edema of the lower extremities. Ascites. Extreme marasmus and death. Autopsy: Ecchymoses beneath the endocardium; slight atheroma of the aorta. Red marrow in the ribs, sternum, small bones of the feet, and femur. Liver showed slight increase of intralobular connective tissue and marked infiltration of lymphoid elements between the lobules and liver cells. Spleen weighed 2,350 grms., and showed hemorrhagic and anemic infarcts; occasional sclerosis of the Malpighian bodies; and increase of reticulum.

*Case 11* (Warren<sup>3</sup>).—Male, age 26. Duration of the disease, at least three months. Diarrhea. Vomiting (no blood mentioned). Regular chills at times ("had not been exposed to malaria"). Enlargement of the spleen. Loss of weight. Dyspnoea on exertion. Moderate anemia of chlorotic type. Marked leucopenia. Splenectomy. The excised spleen weighed 1,155 grms. and showed marked hypertrophy of the reticulum and smallness and irregularity of the Malpighian bodies.

*Case 12* (Hocke<sup>4</sup>).—Female, age 22. Duration, three years. Onset with pain in region of the spleen. Enlargement of the spleen. Hematemesis. Loss of strength. Severe anemia. Leucopenia early; later, leucocytosis. Cervical lymph glands palpable. Autopsy: "Chronic interstitial hepatitis (luecic?);" dilatation of veins at lower end of esophagus and rupture of one of the same; obsolete pulmonary and bronchioglandular tuberculosis; hypoplasia of the genitals. The spleen weighed 700 grms., and showed thickening of the capsule, trabeculae, and reticulum; occasional dilated sinuses; diminution in number and size of Malpighian bodies. (See reference to this and the following case under the discussion of etiology.)

*Case 13* (Chiari<sup>5</sup>).—Male, age 23. Duration, one year. Enlargement of the spleen. Anemia. Very marked ascites with caput medusae. Autopsy: Marked cirrhosis of the liver; dilated veins in anterior abdominal wall and lower part of the esophagus; spleen weighed 600 grms., and showed slight thickening of the capsule, trabeculae, and reticulum with marked fibrosis of the Malpighian bodies. (This case is included here only on the basis of Chiari's diagnosis as splenic anemia, not because such diagnosis could be arrived at from so brief a clinical history.)

*Case 14* (Tansini<sup>6</sup>).—Female, age 46. Duration, 16 months. Onset with abdominal pain and diarrhea, of which there were repeated attacks lasting three to five

<sup>1</sup> *Lancet*, 1904, 2, p. 1013.

<sup>2</sup> *Am. Jour. Med. Sci.*, 1899, 118, p. 428.

<sup>3</sup> *Ann. of Surg.*, 1921, 33, p. 526.

<sup>4</sup> *Berl. klin. Wchnschr.*, 1902, 39, p. 359.

<sup>5</sup> *Prager med. Wchnschr.*, 1902, 27, p. 285.

<sup>6</sup> *Arch. f. klin. Chirurg.*, 1902, 67, p. 874.



days. Enlargement of the spleen and liver. Emaciation. Marked anemia of secondary type. No leucocytosis. No adenopathy; no icterus. Marked ascites. Splenectomy, and recovery. The excised spleen weighed 1,300 grms., and showed marked increase of all connective elements and atrophy of the Malpighian bodies.

*Case 15* (Dock and Warthin<sup>1</sup>).—Female, age 53. Duration, at least six years. Intestinal hemorrhage and diarrhea. Splenomegaly. Ascites. Laparotomy and removal of uterine fibroids, at which operation cirrhosis of the liver was discovered. Edema of the legs. Leucopenia until laparotomy, then leucocytosis. Death from rupture of varicose vein in stomach. Autopsy: Gastric hemorrhages; cirrhosis of the liver; stenosis and calcification of the portal vein with chronic passive congestion of the portal system; anemia; ascites; hyperplasia of the hemolymph glands with excessive hemolysis; peritonitis; hyperplasia of red marrow; tuberculosis of retroperitoneal glands; fat necrosis. The spleen weighed 994 grms., and showed a high degree of fibrosis; marked atrophy of the Malpighian bodies, and a noticeable proliferation of endothelium.

*Case 16* (Hamill<sup>2</sup>).—Male, age 10. Duration, four and a half years. Frequent, sometimes profuse, epistaxis of several days' duration. Skin of a dusky hue since birth but not pigmented. Constant pain in the epigastrium, increased after eating. Hematemesis. Anemia of chlorotic type. Autopsy: Infiltration of the mesentery with dense yellow fat; fibrosis of the pancreas; red marrow in the ribs and tibia. No ulcerations in the stomach nor ruptured varicose veins in the esophagus. The spleen showed moderate atrophy of the Malpighian bodies and "enormous" increase of reticulum.

*Case 17* (Marchand<sup>3</sup>).—Male, age 16. Duration, three years(?). Enlargement of the abdomen with laparotomy for suspected tuberculous peritonitis; much watery fluid evacuated; but peritoneum showed nothing abnormal. Enlargement of the spleen. No enlargement of the superficial lymph glands. Abdominal pain. Severe hematemesis. Marked anemia of a secondary type. Leucopenia early; leucocytosis just before death, which occurred from rupture of varicose vein in stomach. Autopsy: Atrophic cirrhosis of the liver; interstitial pancreatitis; varicose veins in esophagus and cardia; fatty degeneration of the myocardium; fibrinopurulent perisplenitis (slight); edema of the lungs. The spleen weighed 830 grms., and showed marked fibrosis, some "proliferation of the sinuses," and atrophy of the Malpighian bodies.

*Case 18* (Levison<sup>4</sup>).—Male, age 27. Duration, six years. History unimportant except for perforative appendicitis and acute dilatation of the stomach two years before onset of present trouble. The onset was sudden with severe hematemesis. One year later a similar, but more severe attack; another recurrence after four years, and an exsanguinating hemorrhage seven months after this. No pain at any time. Enormous splenomegaly discovered four and a half months after first attack of hematemesis. Marked anemia. Leucopenia except on one count. Ascites. Enlargement of the veins about the umbilicus. General melanoderma. Splenectomy. Recovery with rapid increase in hemoglobin and disappearance of melanoderma. The spleen weighed 1,350 grms. Capsule, and connective tissue throughout the spleen, thickened, especially about the vessels. Much free iron-containing pigment. Malpighian bodies normal; a few undergoing hyaline change. Some proliferating endothelium, "the cells being smaller than such cells usually appear."

<sup>1</sup> *Trans. Assoc. Am. Phys.*, 1903, 18, p. 522.

<sup>2</sup> *Munch. med. Wchnschr.*, 1903, 50, p. 463.

<sup>3</sup> *Arch. f. Pediatrics*, 1902, 19, p. 641.

<sup>4</sup> *Ann. of Surg.*, 1903, 38, p. 671.

*Case 19* (Field<sup>1</sup>).—Female, age 32. Three years before coming under observation, gastric ulcer had been diagnosed. One year before, edema of the lower extremities had developed. Systolic murmur at the apex. Marked anemia of a chlorotic type. Leucopenia. Enlargement of the liver and spleen. Temperature 99 to 100, rising to 105 just before death. Autopsy: Hydrothorax, hydropericardium; purulent bronchitis; atrophic cirrhosis of the liver; punctate hemorrhages in the stomach; interstitial nephritis. The spleen weighed 1,470 grms., and showed marked hyperplastic, interstitial splenitis.

*Case 20* (Clark<sup>2</sup>).—Female, age 19. General weakness. Enormous splenomegaly. Hematemesis and epistaxis. Absence of menses for eight months. Occasional profuse night sweats. Severe anemia of chlorotic type. Sparsely scattered, grayish, pigmented spots on the skin. Systolic murmur at the apex. Shortness of breath and palpitation. Lower edge of liver palpable. Some ascites. Slight temperature. Urine showed trace of albumin. Autopsy: Hydroperitoneum, hydrothorax, hydropericardium; profound anemia; cirrhosis of the liver; dilated and varicose veins in lower part of esophagus; erosion among varicose veins at cardia; blood clot in stomach and intestines; red marrow in tibia. The spleen weighed 3 lbs., 13 oz. Trabeculae and reticulum showed general increase. All vessels congested. Localized areas of extra-cellular pigment. Proliferation of endothelium of small vessels, not of the sinuses. Malpighian bodies few in number.

*Case 21* (Lossen<sup>3</sup>).—Female, age 24. Abdominal pain since five years old: especially after meals. At 12 years, great weakness and hematemesis. Dyspnoea. Cough; no tubercle bacilli in sputum. Great enlargement of the spleen; no glandular enlargement. Systolic murmur (mitral). Slight swelling of the ankles. Severe anemia with low color index. Leucopenia. Splenectomy and death. Autopsy: Peritonitis; sclerosis of the splenic, portal, and mesenteric veins; varicosity of the celiac veins with softened thrombus; soft thrombus in left renal vein; "etat mamillione" of the stomach; red marrow in the ribs and sternum. Spleen weighed 1 kgm. and showed marked thickening of the trabeculae and reticulum; fibrosis of the Malpighian bodies; and cirroid aneurysm of the splenic artery. There was no proliferation of endothelium.

*Case 22* (Standford and Dolley<sup>4</sup>).—Male, age 28. Duration not known. One year previous to coming under observation, an attack of diarrhea and vomiting with blood in vomitus and stools. Later, dragging pains in left side and back. Recent, severe, repeated hemorrhages by stool and from stomach following kick by horse. No pigmentation of the skin. Severe anemia. No leucocytosis. Tumor noticed in lower, middle portion of the abdomen, which laparotomy proved to be an enlarged dislocated spleen. After operation, spleen enlarged rapidly, and 13 days later splenectomy was done. Purpuric spots appeared. Sudden edema of right leg, scrotum, and left side of neck developed. Death 17 days after splenectomy. Autopsy: Interlobular atrophic cirrhosis and chronic passive congestion with beginning central cirrhosis of the liver; chronic passive hyperemia of the rest of the portal system; compensatory hyperplasia of the hemolymph nodes; red marrow in ribs and femur. Marked elongation and torsion of splenic vessels. Spleen weighed 1,650 grms., and showed chronic

<sup>1</sup> *Am. Jour. Med. Sci.*, 1903, 125, p. 495.

<sup>2</sup> *Bristol Medicochirurg. Jour.*, 1903, 21, p. 14.

<sup>3</sup> *Mittteil. u. d. Grenzgeb. Med u. Chirurg.*, 1904, 13, p. 753.

<sup>4</sup> *Am. Jour. Med. Sci.*, 1905, 135, p. 798.

passive congestion and general fibrosis; fibrinous and mixed thrombi in some of the veins; moderate amount of iron-containing pigment; no proliferation of endothelium.

*Case 23* (Strickland, Hodgson, and J Anderton<sup>1</sup>).—Female, age 46. Duration, five years, five months. Eleven children; no miscarriages. First noticed enlargement of the spleen two weeks after last labor, which was normal. Pigmentation of skin excessive. Severe anemia with low color index. Leucopenia. Extreme emaciation and weakness. Melena noticed a few times. Enlargement of the liver with ascites, edema, and jaundice. Death from exhaustion. Autopsy: Stomach, intestines, and bone marrow normal; periportal fibrosis and fatty change in liver; spleen weighed nine pounds, and showed old infarct, general fibrosis, and atrophy of the Malpighian bodies.

*Case 24* (Levy<sup>2</sup>).—Female, age 31. Duration, at least three years. Patient well and healthy until 22 years old, when in six months she lost 25 pounds in weight. In 1893, suffered from chronic diarrhea and pain in left side. In 1895, gave birth to twins; one was stillborn, and the other died in three weeks with rapidly growing, sessile tumors on the posterior surfaces of elbows. Enlarged spleen discovered in 1898. Marked anemia with low color index. Very marked leucopenia. Swelling of the ankles. No ascites. Renal calculi passed. Albuminuria for some months and disappearing later. Large, irregular, dark-brown spots on skin. Death in December, 1902. Autopsy: Purulent peritonitis; enlargement of the mesenteric lymph glands; calculi in right kidney and ureter, which was dilated; spleen weighed more than two pounds, and showed marked increase of reticulum, especially in the pulp, but also in the Malpighian bodies; no proliferation of endothelium.

*Case 25* (Hochhaus<sup>3</sup>).—Male, age 25. Two years before entering the hospital, frequent nosebleed. Later pain in region of spleen and occasional nausea and vomiting. A year later nosebleed recurred often and body assumed slight icteric tint. A few months later hematemesis occurred. In latter part of same year, painful swellings appeared on legs and head which disappeared on treatment with potassium iodide. Ascites. Spleen and liver found enlarged. Urine normal. Moderate anemia with low color index. White blood corpuscles at one count numbered 2,400, later 1,500. Splenectomy; death from hemorrhage. The spleen was very large, and on cut surface showed a yellowish-white circumscribed nodule. Liver somewhat enlarged; at different places on surface, deep, cicatricial contractions. Both organs showed typical microscopic lesions of syphilis.

*Case 26* (Simmonds and Umber<sup>4</sup>).—Male, age 20. For six months, shortness of breath, swelling of the abdomen, and, soon afterward, icterus. Marked ascites recurring rapidly after paracentesis. Enormous spleen. Large, hard liver. Severe anemia; size and form of red cells normal. Number of leucocytes normal. No history or signs of lues. Autopsy: Ascites, cirrhosis of the liver; red marrow; spleen measured 30×20×10 cm.; hard; cut surface of homogeneous appearance with occasional Malpighian body; connective tissues increase moderate.

*Case 27* (F. Bessel-Hagen<sup>5</sup>).—Female, age 26. Abdomen prominent since childhood and continued to enlarge slowly. Abdominal pain and headache frequent. For last two years, swelling of legs. Emaciation. Apex murmur. Slight anemia. No leucocytosis. Prominent veins on anterior abdominal wall. Splenectomy with recov-

<sup>1</sup> *Lancet*, 1904, 2, p. 941.

<sup>2</sup> *Am. Jour. Med. Sci.*, 1905, 155, p. 791.

<sup>3</sup> *Münch. med. Wchnschr.*, 1904, 51, p. 1410.

<sup>4</sup> *Loc. cit.*, p. 28.

<sup>5</sup> *Arch. f. klin. Chirurg.*, 62, p. 212.

ery. At operation liver observed to be smooth but irregularly nodulated. In gastro-colic omentum, stomach, loops of intestine, and peritoneal wall, greatly dilated veins. Spleen normal in shape; weighed 2,506 grms. when removed, later, after blood had escaped, 2,006 grms. It showed general hyperplasia of all the constituents, by far most marked in the reticular connective tissue; Malpighian bodies poorly developed; pigment deposits in the trabeculae.

*Case 28* (J. Roger<sup>1</sup>).—Female, age 50. Great emaciation. Marked anemia with low color index. No leucocytosis. Enlargement of the spleen. Considerable ascites without apparent collateral circulation. No edema of the legs. No mention of hemorrhages. Splenectomy and recovery. The excised spleen weighed 1,650 grms., and showed simple hyperplasia of connective tissue, especially around the vessels.

*Case 29* (J. Roger<sup>1</sup>).—Female, age 35. Duration, 10 months. Emaciation. Progressive enlargement of the spleen. Enlargement of liver. Ascites. Marked anemia with low color index. Leucopenia. Splenectomy with recovery. The spleen weighed 1,270 grms., and showed a condition of "simple hypertrophy." In the spleen pulp were numerous cells containing granular pigment.

*Case 30* (Polloson et Violet<sup>2</sup>).—Female, age 24. At an early age, epistaxis was associated with menstruation. Actual onset of disease unknown. Patient noticed her abdomen enlarged since the age of 12; parents and friends remarked on same peculiarity. Pallor came on only after establishment of menstruation at 13. For more than a year epistaxis had been so abundant as to cause some anxiety; about same time menses began to be abundant and hematemesis occurred. Some edema of the legs. Slight icterus. Moderate anemia. Leucopenia. Urine free from sugar and albumin. Test for alimentary glycosuria negative. Splenectomy with recovery. Excised spleen weighed 1,500 grms. Size diminished markedly with loss of blood from its substance. It was very hard and sclerotic and creaked on cutting. Microscopic anatomy not described.

*Case 31* (Armstrong<sup>3</sup>).—Male, age 26. Catarrhal jaundice at 18 and 20 years respectively. At 21 received a severe blow in left hypochondrium. Afterward vomited dark material twice; and there were localized pain and rigidity. On sixth day after accident spleen found enlarged and firm and continued to enlarge steadily. Slight anemia. Leucopenia; relative number of leucocytes normal. Red cells showed no abnormalities. Coagulation of blood was slow. "Needle punctures bled for some time and the hemorrhage was usually profuse." Occasional diarrhea after slight chilling of surface. Some icterus. Splenectomy, six years after enlargement was first noticed. At operation spleen was found unusually high up and transverse, the position being due to an adhesion at the lower pole. Veins at hilum greatly enlarged; other organs in vicinity normal so far as ascertainable. Spleen weighed 1,000 grms., and measured 22×13×8 cm. Some sinuses dilated and filled with endothelial cells phagocytic for blood pigment. "In many parts the sections present almost no resemblance to splenic tissue, the lymphatic cells being so few in number. Their place is taken by the hyperplastic connective tissue trabeculae and dilated sinuses." Malpighian bodies few in number and of small size; small distinct hyaline masses seen in center of Malpighian bodies.

*Case 32* (Harris and Herzog<sup>4</sup>).—Female, age 22. Duration, nine years. Hematemesis occurring at intervals. Brown pigmentation of the skin. Weakness and loss

<sup>1</sup> *Presse medicale*, 1903, 11, p. 535.

<sup>3</sup> *Brit. Med. Jour.*, 1906, 2, p. 1273.

<sup>2</sup> *Lyon medicale*, 1903, 103, p. 15.

<sup>4</sup> *Annals of Surg.*, 1901, 34, p. 111.

of weight. Chloroanemia. Leucopenia. Splenectomy with recovery. The excised spleen, hardened in formalin, weighed 1,055 grms. and showed large infarct, hyperplasia of all connective tissue elements, some sclerosis of the Malpighian bodies, and moderate proliferation of endothelium.

*Case 33* (Ewart<sup>1</sup>).—Male, age 35. Duration, at least nine months. Death from hemorrhage following rupture of esophageal varix. Spleen weighed 24 ounces, and showed "patchy fibrosis along the trabeculae, little general thickening of the reticulum, slight diminution in the size of the Malpighian bodies, a slight grade of proliferation of endothelium, but no pigment nor giant cells."

#### ETIOLOGY.

Analysis of these cases throws little light on their etiology. Of the 34 patients, 16 were males and 18 females. This differs markedly from Osler's<sup>2</sup> series in which the proportion of males to females was as 13 to 1. Of 29 cases in which the age of onset was given, 10 occurred before 20, 13 between 20 and 40, and 6 after 40 years of age.

A number of theories, based on observations of the above cases, have been advanced to account for the enlargement of the spleen and other manifestations of the disease. Autointoxication from the gastro-intestinal tract (Osler), sclerosis and calcification of the portal vein;<sup>3</sup> syphilis, both congenital<sup>4</sup> and acquired;<sup>5</sup> Leischmann-Dovonan bodies<sup>6</sup> and malaria have all been thought to bear an etiologic relation to this malady.

There is nothing in the history of the case now reported that throws any light on the splenomegaly unless it be the condition found in the liver. Gilbert and Lereboullet<sup>7</sup> believe the enlargement of the spleen is always due to a primary condition of the liver causing obstruction to the portal circulation. They describe three types

<sup>1</sup> Reported by Trevor, *Brit. Med. Jour.*, 1903, 2, p. 576.

<sup>2</sup> *Amer. Jour. M. Sci.*, 1902, 124, p. 751.

<sup>3</sup> Dock and Warthin found this condition in both their cases and thought it had some causative relation to the condition of the spleen. Later work by Warthin (*Amer. Med.*, 1907, 13, p. 532), however, goes to show that artificial obstruction in the splenic or portal vein causes partial atrophy of the spleen, and not hypertrophy.

<sup>4</sup> Marchand, Hocke, Chiari, Hochhaus, *loc. cit.*

<sup>5</sup> Coupland, *loc. cit.*

<sup>6</sup> Marchand, *loc. cit.* See also Marchand and Ledingham, *Lancet*, 1904, 1, p. 140; Leischmann, *Brit. Med. Jour.*, 1903, 1, p. 1252. Donovan, *Ibid.*, 1903, 2, p. 79.

<sup>7</sup> Lereboullet, *Semaine med.*, 1903, 23, p. 180; Gilbert and Lereboullet, *Munch. med. Wchschr.*, 1904, 51, p. 2211.

of cases, all of which are due to a primary angiocholitis with pressure on the intrahepatic branches of the portal vein.

The spleen is the weakest point in the portal system and is the location where passive congestion is most likely to occur first in cases of portal obstruction. Oestereich<sup>1</sup> has called attention to the fact that the splenic enlargement in hepatic cirrhosis may be out of all proportion to the amount of obstruction. The remaining parts of this circulation may show little congestion though the spleen is very much enlarged.

The ease with which the spleen becomes subject to passive congestion has been explained by Weidenreich. The spleen sinuses are wide spaces in the "red pulp" lined by long, slender, fusiform cells with large prominent nuclei (*Stabzellen*). Blood enters these sinuses through the narrow capillaries and leaves through the very narrow "pulp veins." There is thus inserted between the arterial and venous systems of the spleen a series of wide spaces in which the rate of blood flow must be very slow. These sinuses are the weakest points in the vascular system of the organ, and it is here that any backward pressure through the veins from obstruction in the intrahepatic portion of the portal system is most effective. There is not in the human spleen the amount of smooth muscle which Mall<sup>2</sup> found in the dog spleen. Hence the human spleen lacks an important aid in overcoming passive congestion. The *Stabzellen* have some contractile power and unless paralyzed by too great or too long continued over-tension, may assist materially in overcoming stagnation. Toxins of various kinds, malarial for example, are also supposed to paralyze these cells and thus, by this induced hypotonicity, account for the enlargement of the spleen.

Given a very slight obstruction in the intrahepatic portal system, especially if associated with some form of intoxication, and conditions are at once favorable for passive congestion of the spleen, with some degree of enlargement. The passive hyperemia and the toxic substances stimulate connective tissue to proliferation. It is thus, doubtless, that Gilbert and Lereboullet would account for all cases of Banti's disease; and it seems not unreasonable that similar cases may have operated in the present case.

<sup>1</sup> *Virchow's Arch.*, 1895, 142, p. 285.

<sup>2</sup> *Zeitschr. f. Morph. u. Anthropol.*, 1900, 2; *Bull. Johns Hopkins Hosp.*, 1898, 9, p. 218.

## PATHOLOGICAL ANATOMY.

The pathological anatomy of these cases, while not absolutely pathognomonic, shows a remarkable similarity. In all cases the external form of the spleen was retained, the notch on the anterior margin being frequently quite prominent. Adhesions to neighboring viscera were more frequent than Banti<sup>1</sup> originally supposed. The average weight of the spleen was 1,320 grms., the largest weighing nine pounds (about 4,500 grms.).

The spleen microscopically shows marked hyperplasia of connective tissue with more or less passive hyperemia and atrophy of the Malpighian bodies. There was in some cases, a small amount of proliferation of endothelium.

Cirrhosis of the liver was present in 17 out of 24 of the cases,<sup>2</sup> coming on so far as could be determined after the enlargement of the spleen had been noted. Ascites was present in 15 cases. Jaundice was mentioned in five cases, but came on late in the disease. Gastro-intestinal hemorrhages occurred in 21 of the cases.

Varicose veins were present in the lower part of the esophagus and cardia in 10 cases of 26 examined. Seven cases with hemorrhages showed ascites, and of these four had varices in the esophagus and cardia, and three none. In eight cases of ascites without hemorrhages, one showed varices, four showed none, and in the other three splenectomy was followed by recovery, so the presence or absence of varices was not known. These facts are not in complete accord with Preble's<sup>3</sup> results from a study of 60 cases of gastro-intestinal hemorrhage associated with ascites in which he found that "in 6 per cent only of the cases which showed esophageal varices was the cirrhosis typical, i. e., showed ascites, enlarged spleen, and subcutaneous abdominal varices." In two cases with varices gastric hemorrhages were not mentioned; in the remaining eight there was hematemesis. There are thus 13 cases of gastro-intestinal hemorrhage in which there were no varices found. Taking from these four cases in which successful splenectomy was done, there are still left nine cases of gastric hemorrhage in which no distinct lesion was mentioned in the stomach. These cases

<sup>1</sup> Ziegler's *Beiträge*, 1898, 24, p. 21.

<sup>2</sup> There were 10 cases of successful splenectomy in which the condition of the liver was not determined.

<sup>3</sup> *Am. Jour. Med. Sci.*, 1900, 119, p. 263.

seem to conform to Osler's<sup>1</sup> statement that the cause of the hemorrhage in Banti's disease is to be found in the spleen and not in the liver. In five cases showing the combination of hematemesis, cirrhosis, and varices, the disease had lasted four, three, one, six, and three years respectively. If allowance is made for the insidious onset and for the fact that the enlarged spleen may have existed for some time before being discovered (the number of years of duration usually represents the time from the discovery of the splenomegaly to death or operation), it is easy to conceive that these cases may have passed into the third stage of the disease as originally described by Banti. The earlier hemorrhages may thus have been due to conditions in the spleen, the later ones to conditions in the liver, which caused the varices.

Those cases of gastric hemorrhages without discoverable lesion in the stomach arise from congestion of the gastric mucosa due to obstruction to outflow of blood and "simultaneous rupture of many capillaries of the gastric mucous membrane." Mall<sup>2</sup> has shown that 40 per cent of the blood from the stomach reaches the portal system by way of the vasa brevia and splenic vein. Hence congestion in the splenic system of veins, whether due to obstruction in the intra-hepatic portal branches or to some more localized cause, will produce congestion in the wall of the stomach. "The stress of the congestion is continuously felt in the submucous capillary system, and the hemorrhage, which is apt in such cases to occur from the loaded membrane; receives a simple solution upon principles almost purely mechanical" (Watson<sup>3</sup>).

#### SYMPTOMATOLOGY.

The symptomatology, as illustrated by these cases, may be briefly discussed. A progressive anemia with low color index, with no leucocytosis or with leucopenia, associated with enlargement of the spleen, are the only constant symptoms. Other less common symptoms in the order of their frequency are: gastro-intestinal hemorrhages, ascites, diarrhea, epistaxis (eight times), pigmentation of the skin (seven times), and jaundice, which occurs late in the disease.

<sup>1</sup> *Am. Jour. Med. Sci.*, 1902, 124, p. 751.

<sup>2</sup> Quoted by Osler, *Am. Jour. Med. Sci.*, 1902, 124, p. 751.

<sup>3</sup> Quoted by Osler, *loc. cit.*



## GROUPING OF CASES. II.

Another group of cases presenting a markedly different anatomical picture and somewhat different clinical manifestations is found in the literature under the name of "Banti's disease," "primary splenomegaly," "splenic anemia," or synonymous terms. These cases are characterized anatomically by enormous, diffuse proliferation of the endothelium of the spleen, and sometimes of the retroperitoneal lymph glands and liver. This condition shows a peculiar tendency to occur in certain families. It is characterized clinically by a chloroanemia; absence of leucocytosis; enlargement of the spleen; in nearly all cases, hemorrhages from the nose, gums, or under the skin; and, less frequently, jaundice.

Little attention seems to have been paid by writers on this subject to the possibility of a clinical differentiation between the two groups of cases, although all have been greatly impressed by the marked anatomical differences. In 1901 Brill<sup>1</sup> published the clinical histories of two cases. He concluded from a study of these patients that the anatomical basis of their disease was identical with that described by Bovaird,<sup>2</sup> in which the spleen showed diffuse proliferation of endothelium. Post-mortem examination of one of Brill's<sup>3</sup> cases four years later proved the correctness of his conclusions. More recently Schlagenhauser<sup>4</sup> has called attention to the distinguishing clinical characteristics of this condition with the terms the "Gaucher type of splenomegaly."

## SUMMARY OF REPORTED CASES.

*Case 1* (Gaucher's).—Female, age 32. Duration of disease, 25 years. Gradually developing, symmetrical enlargement of the spleen with secondary enlargement of the liver, and jaundice. Remarkable proneness to hemorrhages in the skin and from the nose and gums. Anemia. No leucocytosis. No glandular enlargement nor ascites. Death from increasing cachexia. The spleen weighed 4,770 grms. Microscopical examination showed slight hyperplasia of the trabeculae and capsule; thickening of the walls of the blood vessels; disappearance of the Malpighian bodies; and, in the meshes of the reticulum, cells with large nuclei and a large or small amount of protoplasm, which Gaucher called "spleen epithelium." He called the condition a primary epithelioma of the spleen. The liver showed a primary interstitial hepatitis without contraction and without the formation of new bile ducts.

<sup>1</sup> *Am. Jour. Med. Sci.*, 1901, 121, p. 378.

<sup>2</sup> *Am. Jour. Med. Sci.*, 1905, 129, p. 491.

<sup>3</sup> *Am. Jour. Med. Sci.*, 1900, 120, p. 377.

<sup>4</sup> *Virchow's Arch.*, 1907, 187, p. 125.

<sup>5</sup> *Splenomegalie primitive*, Thèse, Paris, 1882; and *Semaine med.*, 1892, 12, p. 331; abstract in *Schmidt's Jahrb.*, 1892, 236, p. 137.

*Case 2 (Collier<sup>1</sup>).—Female, age 6. Duration of the disease, four years. Abdomen began to enlarge at the age of two years. Excessive emaciation. Slight beading of the ribs but no other signs of rickets. Red blood cells misshapen and did not form good rouleaux. Leucocytes were not increased in number. Temperature usually subnormal. "Death seemed to be accelerated by an attack of epistaxis and sickness." The spleen weighed 4 lbs., 2 oz. Microscopic examination showed the reticulum replaced by very large endothelial cells, in places filling the splenic sinuses completely. Malpighian bodies could not be distinguished. Slight increase in thickness of trabeculae in places. A similar condition was found in some of the retroperitoneal glands.*

*Case 3 (Bovaird<sup>2</sup>).—Female, age 16. Duration, 13 years. Abdomen began to enlarge at the age of three, with hard mass in left side. Slight enlargement of the superficial lymph glands. Pigmentation of the skin similar to that in Addison's disease. Slight temperature at times. Some albuminuria. Severe anemia of secondary type. No leucocytosis. Splenectomy. Death. Spleen weighed 12½ pounds (total weight of child not more than 75 pounds). There were large anemic infarcts in the spleen. Microscopically the venous spaces were dilated, and in places filled with large cells with relatively small, variably staining nuclei. Many of these cells lay free in the spaces; others were attached to their walls. Numerous giant cells were present. Capsule and trabeculae thickened; an increase of perivascular connective tissue. Malpighian bodies showed little change; in places they had been encroached upon by the proliferation of endothelium of the pulp. Lymph glands at the hilum of the spleen showed hyperplasia of the endothelium and a peculiar deposit of iron-containing pigment in the periphery of the follicles. In the liver, there were small, strictly localized areas of hyperplasia of endothelium, which Bovaird thought originated *in situ* and not as metastases.*

*Case 4 (Brill<sup>3</sup>).—Male, age 23. Duration, 13 years. Spleen greatly enlarged. Marked anemia with low color index. Leucopenia. Epistaxis, petechiae, and ecchymoses late. Yellowish-brown discoloration of the skin on exposed parts. Urine negative. Enlarged liver, but no ascites until two days before death. No edema. Patient always felt well except during an intercurrent infection. Autopsy: Fibrinohemorrhagic pericarditis. Perisplenitis and perihepatitis. Chronic interstitial hepatitis with presence of proliferating endothelium. Hemorrhages in mucosa of ileum and colon. Red marrow in femur containing abundant endothelial cells. Lymphoid tissue of peribronchial, mesenteric and retroperitoneal lymph nodes almost completely replaced by endothelial cells. Spleen weighed 5,280 grms., and showed old and new infarcts; slight increase of connective tissue; very extensive proliferation of endothelium; little or no change in the Malpighian bodies.*

*Case 5 (Hawkins and Seligman<sup>4</sup>).—Male, age 37. Duration, at least three months. General weakness. Enlargement of spleen and liver. Slight jaundice. Irregular temperature. Night sweats. Epistaxis and bleeding from the gums late. Secondary anemia. Leucopenia. Recurring diarrhea. Systolic murmur in pulmonary area. Some edema of shins; no ascites. Autopsy: Subpericardial hemorrhages and numerous petechiae in the myocardium. Vegetative mitral and aortic endocarditis. Focal necrosis containing bacteria and cloudy swelling of the liver; no cirrhosis. Spleen weighed 25 oz., and showed infarct; focal necrosis; slight fibrosis*

<sup>1</sup> *Trans. London Path. Soc.*, 1806, 46, p. 148.

<sup>2</sup> *Am. Jour. Med. Sci.*, 1905, 129, p. 491.

<sup>3</sup> *Am. Jour. Med. Sci.*, 1920, 120, p. 377.

<sup>4</sup> *Lancet*, 1903, 1, p. 787.

affecting the trabeculae and sheaths of the vessels; and considerable evidence of proliferation of endothelium.

*Case 6* (Picou et Ramond<sup>1</sup>).—Female, age 32. Duration, four years. Onset insidious with vague abdominal pains, enlargement of the spleen and later of the liver. Anemia without leucocytosis. Severe menorrhagia. Later, hemorrhagic diathesis with scorbutus-like changes in the gums. No ascites. No enlargement of the superficial lymph glands. Operation followed by only moderate improvement. The excised spleen weighed 2,800 grms. The greater part of its substance was replaced or destroyed by alveolar cell masses from the arrangement of which the authors called the condition a primary epithelioma of the spleen of the Gaucher type.

*Case 7* (O'Malley and O'Malley<sup>2</sup>).—Male, age 18. Duration, at least eight months. Enormous splenomegaly. Epistaxis occasionally; bleeding from the gums daily. No hematemesis. Occasional diarrhea. Pigmentation of the skin. Superficial lymph glands palpable. Leucopenia, except during one month. Severe anemia. Color index constantly greater than 1.00, due to hemoglobinemia as demonstrated by spectroscopy. Splenectomy. Death. Autopsy: Septic peritonitis. Parenchymatous nephritis. Atrophic cirrhosis. The spleen weighed 945 grms., and showed some connective tissue hyperplasia; marked proliferation of endothelial cells which completely filled most of the sinuses; giant cells with both central and peripheral nuclei; and enlargement of the Malpighian bodies due to proliferation of endothelium within them.

*Case 8* (Springthorpe and Stirling<sup>3</sup>).—These authors reported six cases in one family. *Case 1*. Female, age 29. Typhoid fever at eight; "bilious attacks" and vertigo till 15 years of age. Spleen first found enlarged at 25. Severe anemia. Dull, paroxysmal pain over spleen, liver, and kidneys. Attacks of vomiting lasting for eight or nine days, the vomitus containing blood. Epistaxis twice. Splenectomy. Complete recovery. The excised spleen weighed 43 $\frac{3}{4}$  oz. Sections showed much pigment; decrease in the number of Malpighian bodies and lymphoid elements; thickening of the vessel walls; and a "great increase of the fibrillar framework of the spleen pulp, made up of anastomosing processes of endothelial cells with their rounded or elongated nuclei."

*Case 9* (Umbert<sup>4</sup>).—Male, age 15. Anemia and large spleen for eight years before coming under observation. Patient felt well until summer of 1903 when there occurred headache and, for one week, nosebleed almost continuously, and slight icterus. Entered hospital in December, 1903, at which time his condition was as follows: Slight icterus. Enormous, hard, smooth tumor of the spleen. Liver symmetrically enlarged. No ascites. Appetite good. Chest organs normal. Stools gave positive reaction (Weber's) for blood. Secondary anemia. Leucopenia. Condition of patient grew steadily worse and during the next month slight ascites was demonstrable on percussion. Later, splenectomy was performed, at which time no ascites nor enlargement of the mesenteric glands was apparent. The spleen weighed 1,300 grms. Sections showed numerous deposits of pigment; enormous overfilling of the sinuses with blood; numerous mononuclear cells with fine pigment granules; in the Malpighian bodies, numerous giant cells similar to those found in bone marrow. The splenic veins were normal. The walls of the arteries frequently showed hyaline degeneration. Increase of connective tissue was not perceptible. A bit of liver removed at operation

<sup>1</sup> *Arch. gen. de Med.*, 1896, 168; Abstract in *Hildebrand's Jahresb.*, 1896, 2, p. 836.

<sup>2</sup> *Am. Jour. Med. Sci.*, 1905, 129, p. 996.

<sup>3</sup> *Lancet*, 1904, 2, p. 1013.

<sup>4</sup> *Ztschr. f. klin. Med.*, 1904, 55, p. 289.

showed accumulations of cells, chiefly lymphocytes, in the periportal connective tissue. The parenchyma was perfectly normal.

*Case 10* (Harris and Herzog<sup>1</sup>).—Male, age 47. Duration about eight months. Typhoid at three years, possible malaria at 30. Dull pains in epigastric and umbilical regions. Splenomegaly. Anorexia. Nausea, vomited once, but no blood. Loss of weight. Anemia with low color index. Leucopenia. Splenectomy with recovery. The spleen, after being fixed in formalin, weighed 600 grms., and showed some hyperplasia of connective tissue elements, some sclerosis of Malpighian bodies, very marked proliferation of endothelium, and abundant infiltration with hematoidin and hemosiderin.

*Case 11* (Rolleston<sup>2</sup>).—Male, age 23. Duration, 12 years. Hematemesis for three years. Epistaxis. Splenomegaly. Anemia of chlorotic type. Leucopenia. No enlargement of the liver and no jaundice. Urine normal. Splenectomy; death on second day from gastro-intestinal hemorrhage. No autopsy. Spleen weighed 37 oz., and showed fibrosis, widespread proliferation of endothelium and disappearance of leucocytic elements.

*Case 12* (Williamson<sup>3</sup>).—Male, age 9. Duration, two years. Epistaxis. Severe anemia of chlorotic type. Leucopenia. Splenomegaly. Splenic pain. Enlargement of the liver. Slight edema of the feet; no ascites. No enlarged glands. Autopsy: Mitral endocarditis. Ulceration of the intestine. Peritonitis. Bronchopneumonia. Red marrow in the sternum and humerus. Slight cirrhosis of the liver. The spleen weighed 2 lbs., 7. oz., and showed increase of connective tissue, some fibrosis of the Malpighian bodies, and "enormous numbers of large nucleated cells, each containing 6 to 10 red blood corpuscles." (These cells were in the pulp sinuses and were probably proliferating endothelium.)

*Case 13* (Stengle<sup>4</sup>).—Female, age 21. Probable onset years before coming under observation. Two stillborn children. Enlarged spleen. Palpable superficial lymph glands. Pain in left side. Slight anemia. Eosinophilia, average of 7 different counts, 6.2 per cent; highest counts, 10.5 and 11 per cent respectively of eosinophiles. Splenectomy with recovery. After hardening, the spleen weighed 1,230 grms. The surface was covered with numerous umbilicated projections which were seen on section to be the apices of "masses of growth" inside the spleen. Sections of these masses showed enormous proliferation of endothelium, giving almost the appearance of an infiltrating tumor mass. The pulp tissue in these involved areas was small in amount. The Malpighian bodies were frequently atrophied and sometimes hyaline.

*Case 14* (Schlagenhauser<sup>5</sup>).—Female, age 43. Duration, 38 years. Enlargement of the spleen noticed since five years of age. Liver enlarged. No ascites. Enlargement of submaxillary glands. Brown pigmentation of the skin. Very marked leucopenia—blood count at one time showed only 800 leucocytes. Marked reduction of the hemoglobin with a slight diminution in the number of red cells. Hemorrhages from nose and gums. Death from suppurative cholangitis following gall stones. Post-mortem examination showed suppurative cholangitis and cholelithiasis; tuberculosis of lymph nodes, liver, bone marrow, and spleen; red marrow in long bones; blood-tinged material in stomach and intestines. The spleen weighed 3,510 grms. Microscopically, it showed (in the parts not tuberculous) the blood sinuses containing

<sup>1</sup> *Ann. of Surg.*, 1901, 34, p. 111.

<sup>2</sup> *Clin. Jour.*, 1902, 10, p. 401.

*Med. Chron.* (Manchester), 1893, 18, p. 103.

<sup>4</sup> *Am. Jour. Med. Sci.*, 1904, 128, p. 497.

<sup>5</sup> *Virchow's Arch.*, 1907, 187, p. 125.

large numbers of "liver-like" cells, some free in the sinuses, some attached to their walls, with small, deeply staining nucleus and pale protoplasm. The Malpighian bodies were reduced in numbers. Proliferation of endothelium was also present in the liver, lymph nodes, and bone marrow.

Schlagenhauser also gives a brief clinical history of a sister of the above patient who was similarly affected.

#### ETIOLOGY.

In this group of cases, there is a remarkable tendency for several cases to occur in the same family (Bovaird, Brill, Springthorpe and Stirling, and Schlagenhauser). Of the 14 cases, seven were males and seven females. The average age of the patients at the onset of the disease was 15 years. In 10 cases the onset was before 20, in three between 20 and 40; and in one after 40 years of age.

Many investigators, notably Stengel,<sup>1</sup> believe that this anatomical condition "represents a primary neoplasm of the spleen somewhat comparable to diffuse myelomata and infiltrating sarcomata of the liver." But the following facts speak against its being of neoplastic origin: (1) The proliferation of endothelium, in almost all cases, is uniformly distributed throughout the spleen giving no appearance of having originated in one focus and of having spread to other parts of the spleen by continuity of tissue. One of Stengel's cases is an exception to this statement. (2) Bovaird, who made a most elaborate study of the anatomical changes, concluded that the proliferating endothelium in the liver and lymph nodes originated *in situ* and not as metastases from the spleen. (3) The long duration and the perfect cure after successful splenectomy render a malignant character extremely doubtful.

It seems more reasonable to consider the pathological process in the spleen to be of an inflammatory nature. It will be recalled that Mallory<sup>2</sup> has shown that the essential lesion in typhoid fever is a proliferation of endothelium, which even acquires almost a malignant character for a time; which is limited almost entirely to Peyer's patches and the solitary lymph nodes of the intestine, the mesenteric lymph glands, and the spleen; and which is induced by the circulating typhoid toxin. Adami, Abbott, and Nicholson<sup>3</sup> found swelling of, and phagocytic action by, the endothelial cells of the liver of rabbits within a few minutes after injection of a culture of colon bacilli.

<sup>1</sup> *Prog. Med.*, 1905, 7, p. 240.

<sup>2</sup> *Jour. Exp. Med.*, 1898, 3, p. 611.

<sup>3</sup> *Jour. Exp. Med.*, 1899, 4, p. 362.

The fact that in the condition now under consideration, proliferation of endothelium may originate *in situ* in the liver and retroperitoneal lymph nodes is strongly suggestive that the real cause is not purely local in its action, though it may possibly be local in its origin.

#### PATHOLOGY AND SYMPTOMATOLOGY.

The normal shape of the spleen is retained. The average weight of the spleens in the above cases was 2,300 grms. The largest weighed 12½ pounds (Bovaird).

The anatomical changes in the spleen have been too thoroughly described by Bovaird to require repetition in this place. Suffice it to say that the endothelium lining the splenic sinuses was markedly proliferated and frequently filled the sinuses completely. Proliferating endothelium was found in the liver and retroperitoneal glands in three cases (Bovaird, Brill, and Schlagenhauer).

Cirrhosis of the liver, not of extreme grade, was present in three cases, being in one of them associated with proliferation of endothelium. Ascites occurred twice—in one case (Brill's) only two days before death; in the other (Umber's) it was found at an exploratory laparotomy and later disappeared. Jaundice was present four times; and pigmentation of the skin other than jaundice, four times. Diarrhea was mentioned in three cases. Gastro-intestinal hemorrhages occurred three times. The hemorrhages in these cases took the following forms in order of frequency: epistaxis (nine times); bleeding from the gums (five times); severe menorrhagia (once).

One of the cases of Dock and Warthin, one of Stengel's, and Jaffrey's could not be satisfactorily brought under either of these groups. They showed symptoms common to both groups, and in the spleen proliferation of connective tissue and of endothelium were both present. They could not be classified on that basis.

Cases have been reported by Grestel,<sup>1</sup> Mueller,<sup>2</sup> Strümpell,<sup>3</sup> Landouzy,<sup>4</sup> Lodi,<sup>5</sup> Glockner,<sup>6</sup> Peacocke,<sup>7</sup> Marchand,<sup>8</sup> West,<sup>9</sup> Quenu

<sup>1</sup> *Berl. kl. Wchnschr.*, 1866 5, p. 212.

<sup>2</sup> *Ibid.*, 1867, 6, p. 434.

<sup>3</sup> *Arch. der Heilk.*, 17, 18; quoted by Bruhl, *Arch. gen. de med.*, 1891, 2, p. 160.

<sup>4</sup> *Bull. Soc. Anat.*, 1873; quoted by Bruhl.

<sup>5</sup> *Traité de la Leucémie*, Bologne, 1880; quoted by Bruhl.

<sup>6</sup> *Munch. med. Abhandl.*, 2. Reihe, 11. Heft, 1895.

<sup>7</sup> *Dublin Jour. Med. Sci.*, 115, p. 274; see also *Lancet*, 1903, 2, p. 748

<sup>8</sup> *Munch. med. Wchnschr.*, 1903, 50, p. 493.

<sup>9</sup> *Medico-chirurg. Trans.*, 1896, 79, p. 323; for criticism of this case see Kanthack, *Brit. Med. Jour.*, 1896, 2, p. 719.

and Duval,<sup>1</sup> Thiel,<sup>2</sup> and Borissowa,<sup>3</sup> either under the diagnosis of Banti's disease or have been referred to by other writers on this subject as belonging to this category. But either the data given were too meager to justify any conclusions based on them or there seemed to be good reason for doubting whether certain of these cases properly belong in the same class with the foregoing two groups.

#### SUMMARY AND CONCLUSIONS.

While there may be some just question whether all the cases here collected are properly classified, there can be no doubt that there are two distinct conditions associated with an idiopathic anemia and enlargement of the spleen. One begins usually in patients over twenty years of age; is characterized clinically by chloroanemia, leucopenia, enlargement of the spleen, and quite frequently by gastrointestinal hemorrhages, ascites, pigmentation of the skin, very rarely by jaundice; and anatomically shows fibrous hyperplasia of the spleen with, frequently, cirrhosis of the liver, and varicose veins in the lower esophagus and cardia. The other occurs most often in young people and shows a family tendency; manifests itself clinically by an anemia with low color index, absence of leucocytosis, enlargement of the spleen, a prolonged course, hemorrhages from the nose and gums or under the skin and mucous membranes, and, less frequently, by jaundice and brownish pigmentation of the skin; and is characterized anatomically by diffuse proliferation of endothelium in the spleen and sometimes in the liver and retroperitoneal lymph glands.

I wish to express my thanks to Dr. E. R. LeCount for suggesting this work and for advice in the prosecution of it; and to Dr. Knap for photomicrographs of sections from the case reported.

<sup>1</sup> *Rev. de Chirurg.*, 1903, 28, p. 444.

<sup>2</sup> *Deut. Ztschr. f. Chirurg.*, 1906, 84, p. 576.

<sup>3</sup> *Virchow's Arch.*, 1903, 172, p. 108.

## ON THE SIMILARITY BETWEEN BLOOD-PLATELETS AND CERTAIN HEMATOZOA.\*†

(WITH PLATE I.)

LEROY D. SWINGLE.

It is a well-known fact that students of hematozoa have from time to time announced the discovery of new blood-parasites, and later have found them to be only normal constituents of the blood. Being aware of this fact and prompted by recent investigation upon *Babesia* (*Piroplasma*), I am led to make a statement of certain facts which have come under my observation in the course of investigations on a flagellate occurring in the stomach of the sheep tick, *Melophagus ovinus*.

The mode of transmission of *Babesia* has been a matter of discussion ever since the discovery of the parasite. Schaudinn's (1904) hypothesis that the development takes place in a similar manner to *Halteridium* has been well supported, not only by his own observations, but by experiments of other workers. He states that he found on blood films prepared late in the afternoon by Weber and Kossel from a cow kept in the dark and dying of piroplasmosis, flagellates in which both a blepharoplast and a nucleus could be made out. He then restained and studied old streak preparations made in 1899 from the intestinal contents of ticks which had fastened themselves to hemoglobinuric oxen. Trypanosoma-like stages similar to the above were found.

Working on this hypothesis, Rogers (1904) found that flagellates developed in cultures made by putting blood of patients suffering from certain cachexial fevers and kala-azar into sodium-citrate solutions. His best flagellate stages were obtained in one culture after an incubation of 24 hours at a low temperature. Judging from his figures alone, one could hardly doubt that they represent real trypanosome stages. Still stronger evidence for the existence of a flagellate stage in some insect host is afforded by the statement in a later paper (1905), that an acid solution similar to the conditions existing in the stomach of certain insects is the most conducive to the development of flagellates.

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In a very recent paper (1907) he seems to have shown conclusively that the parasite causing kala-azar does have a flagellate stage. From sodium-citrate cultures he obtained rosettes of dividing flagellates with long flagella centrally located. When the cultures were first made the parasites were small and it was hard to distinguish them from blood-platelets. But later, growth and flagellation took place and by successive divisions the rosettes originated.

Kinoshita (1907) in a paper on *Babesia canis* draws comparisons between this parasite and *Plasmodium*. He claims, although there is room for some doubt, to have found schizogony and gametogony, and lays considerable emphasis upon the presence of nuclear dimorphism. Furthermore, in sodium-citrate cultures he found flagellates, the flagella of which took origin in the blepharoplast and measured in one case  $15\ \mu$  in length.

The works of Koch (1906), Kleine (1906), and Christophers (1907), do not support the hypothesis of a flagellate stage. Further mention of Koch's and Kleine's papers will be made later. Christophers followed the life-cycle of *Piroplasma canis* through the tick, but neither mentions nor figures any flagellate stage. It is even more striking that he says nothing about nuclear dimorphism.

According to the observations of Fantham (1907) upon *Piroplasma bigeminum* no flagellate stage occurs, although nuclear dimorphism is clearly present. He says, however, "It is most probable, indeed certain, that a flagellate stage does occur in the life-cycle of the Leishman-Donovan body, and may be expected in the alimentary tract of a blood-sucking Arthropod, namely, the bedbug, as suggested by Rogers and now being worked out by Patton. On the other hand, a flagellate stage appears to be absent in the case of *Piroplasma*."

It seems rather strange that the results of these observers should be so contradictory. Furthermore, it would appear quite improbable that, as Fantham suggests, the Leishman-Donovan body would pass through such an important phase as a flagellate stage, which is not to be found in *Piroplasma*, provided that the two forms are very closely related, as is generally believed.

A statement of my own experience may possibly throw light upon the subject. In my investigation of a herpetomonadine flagellate existing in the alimentary tract of the sheep tick, the mode of trans-

mission from tick to tick at once became a question. The fact that insects are known to be intermediate hosts for various other flagellates suggested the possibility that the sheep tick might act as an intermediate host in this case, and, therefore, that the parasite ought to be found in the blood of the sheep. Accordingly, examinations of the sheep's blood were made, but always with negative results. Knowing Schaudinn's hypothesis and the results of Rogers' experiments, the thought occurred to me that I might be dealing in the tick with the flagellate stage of a piroplasma-like form, possibly *P. ovis*, which I had overlooked in the sheep. Working on this hypothesis, I proceeded to make sodium-citrate cultures of sheep's blood, and was gratified to find that flagellates developed which were quite similar to young stages found in the tick. The same results were obtained from different sheep, both young and old. For a check, cultures of dog's, rabbit's, and human blood were made, giving almost identical results. This led me to a more thorough investigation of the origin of these flagellates, resulting in the discovery that they were nothing more than blood-platelets (thrombocytes). A detailed description of these elements will serve to show the possibility of mistaking them for a flagellate stage of *Babesia* or some other species.

The sodium-citrate solution was made by adding to 1,000 c.c. of distilled water, 5 gm. sodium citrate and 5 gm. common salt. Later it was found, as Rogers also noticed in his cultures, that an acid reaction gives the best results. And so, after sterilization, the solution was rendered slightly acid by the addition of a few drops of hydrochloric acid.

The part of the animal from which the blood was to be drawn was thoroughly washed, in some cases with sublimate, in others with strong alcohol, and then clipped with sterile scissors or punctured with a needle. One or two drops of the blood were allowed to drop directly into a test-tube containing about 2 c.c. of a sodium-citrate solution.

The cultures were examined as quickly as possible after the blood was drawn, generally within five or ten minutes. Normal blood-platelets measuring about one-third the diameter of red blood corpuscles appear from surface view circular, oval, or somewhat ameboid, while from a view at right angles to this they appear spindle-shaped, showing that they are biconvex. It should be noticed that these

normal platelets resemble normal piroplasma forms in size and general shape. A nucleus, and refractive spots, probably vacuoles, can be seen (Figs. 1, 2, and 3). For a more detailed description of the normal platelet and its behavior in blood-clot, reference can be made to the works of Dekhuyzen (1901) and Kopsch (1901). In ordinary blood smears, generally all that can be seen of the platelets is a little granular nuclear material. But after they have been in sodium-citrate solution they can readily be fixed on a slide and stained. My method was to place a drop of the culture upon a slide and after it had evaporated down, but was not entirely dry, to drop on some killing fluid, Zenker's being used with the best results. The specimens could then be stained according to Wright, Giemsa, or with iron hematoxylin.

Cultures examined as quickly as possible after drawing the blood already showed ameboid and flagellate forms. A fact of great importance to the student of hematozoa is that the most of these forms are capable of movement. They were seen to roll over, swing round, and often move for a distance equal to the diameter of two or three red corpuscles. Among the ameboid types there were always present in great numbers platelets with few or several long, sharp, or sometimes blunt, pseudopodia (Figs. 17-24). From the description and figures of Koch and Kleine one must conclude that these forms are very similar to, if not identical with, what they describe as developmental stages of *Piroplasma*. To be sure, Koch found his stages in the stomach of the tick, but this does not disprove the statement, for just such forms were also found in the stomach of the sheep tick after sucking the blood of a sheep. Another form which is often found and which has been figured by Dekhuyzen is spindle-shaped with a long, stiff process at one end and a shorter one at the other (Fig. 4). Many times these forms were somewhat bent, appearing like a quarter-moon. In older cultures most of the platelets that had no pseudopodia were at the rim, either inside or just outside, of a transparent circle about the size of or a little larger than the platelet. Those outside looked as if they had crawled out of a thin envelope. As to their significance, I can only say that they apparently were not degeneration forms, inasmuch as they were still active in their ameboid movements.

The more typically flagellate forms, those with a single flagellum, are perhaps of still more importance, because they so perfectly simulate real flagellates. Although they are found immediately after the introduction of the blood into sodium-citrate solution, the "flagellum" is generally quite rigid except at the very end, where it can be seen to vibrate. Notwithstanding this rigidity, they are seen to move about, roll over, and swing around, these movements probably being the result of the vibrations at the tip of the flagellum. The most motile forms were found in a culture of human blood kept for the first six hours in an ice chest and after that at room temperature for 50 hours. Round or pear-shaped individuals with a flagellum measuring in some cases as much as  $20\mu$  were found in abundance (Figs. 5-12). In the pear-shaped forms the flagellum is at the pointed end. It was very slender, in most cases appearing and moving very much like the flagellum of *Euglena*, often with lashings violent enough to move the red blood corpuscles on coming in contact with them. Instead of being smooth, in some instances the flagella had thickened, knotted portions, which bear a close resemblance to Kinoshita's description and drawings of the flagellates which he found (Figs. 5 and 18). It is important to note that he found the best-developed flagellate with a flagellum  $15\mu$  long in sodium-citrate culture.

The various forms are often found grouped together in couplets, triplets, or in masses composed of as many as a hundred individuals. In this condition they retain their individual motion, rolling over and turning about. I have seen these masses stained with iron hematoxylin so that they had the exact appearance of Kleine's photograph (Kleine, Pl. IV, Fig. 14).

Such masses as these have often been found in the stomach contents of sheep ticks which have fed on sheep's blood. The individuals sometimes were seen to separate and move about, their flagella then becoming plainly visible. Single forms with long waving flagella exactly similar in size, shape, and motion to the platelets in sodium citrate were found. Then, again, the spindle-shaped, moon-shaped, and ameboid forms with several long, stiff processes were observed. Just such a form as represented by Koch (Pl. I, Fig. 14) was found not only in the tick but also in sodium-citrate culture. To eliminate the possibility of confusing the platelets with the herpetomonadine

flagellates, which are generally present in adult ticks, they were studied in young ticks before the latter had become infected.

Flagellation of blood-platelets is not limited to sodium-citrate culture, but may take place in other media. As already mentioned, it occurs in the stomach of the sheep tick; it will take place in sodium citrate without an acid reaction; no less certainly, though perhaps not to the same degree, will it occur in ordinary normal salt solution; and, finally, some of my best results were obtained from a sodium-citrate culture to which was added one-eighth agar-agar. In this last culture a type more definite and constant in structure was found along side the types described above. Unfortunately, however, I overlooked them in the living condition, and did not find them till I examined permanent mounts made from the culture after an incubation of 24 hours. This type is more or less rounded, and has one flagellum originating at a definite point in a kind of notch formed by a little curved projection (Figs. 14-16). In some cases I thought I could recognize features transitional between this type and the other monoflagellate forms. On the average they are larger than the other forms. There is no such variability among them in size and shape as is found in the blood-platelets. The relative position of the chromatin masses in the body is quite constant. With the data at hand, no final opinion is possible, yet the evidence points toward a contamination with monads.

In stained preparations one often finds a most striking, yet doubtless merely coincident, resemblance in nuclear conditions to trypanosome forms. While nuclear dimorphism is not by any means to be found in all blood-platelets, yet it is by no means rare. And when it does occur there is not such marked distinction between the nuclear masses as one sees in trypanosomes, but still as much distinction as many of Kinoshita's drawings would indicate for *Babesia*, in which, he claims, nuclear dimorphism comparable to nucleus and blepharoplast of the trypanosomes is present (compare my figures with his Pl. XII). In blood-platelets a condition exists which should be considered a *false* dimorphism as compared with the trypanosomes or *Babesia*. Thus, it would be an easy matter to mistake such blood-platelets for real flagellates having *true* nuclear dimorphism. Forms represented by Figs. 10 and 17 could be explained by regarding them as division

stages in which the nucleus and "blepharoplast" had divided, interpreting the smaller nuclear masses as blepharoplasts and the larger as nuclei.

The flagella and pseudopodia stain like cytoplasm, and not like chromatin as in the trypanosomes. Koch states that in his forms they stain like cytoplasm, while Kinoshita says his stain like chromatin. If Koch and Kinoshita are both correct in their statements, the evidence indicates that Kinoshita observed a true flagellate form, and further that a possible identity exists between Koch's forms and blood-platelets.

One needs only to compare my drawings with Koch's and Kleine's to be convinced of their similarity in size, shape, and structure. Koch gives no measurements and Kleine only gives measurements of some exceptionally large forms, which, pseudopodia expected, measured  $14\ \mu$  long by  $4\ \mu$  broad. The latter are larger than any forms that I found. The scale present on my plate will give the reader a more correct idea of the size than could be given by the use of numerals in the context. In comparing plates it should be noted that my drawings are magnified 800 diameters more than Koch's and Kleine's. In Kleine's photographs one will see that some of the pseudopodia are rigid and blunt, while others are wavy and tapering. (Compare my Figs. 4-24 with his Pl. IV, Figs. 5-14, also Pl. V, Figs. 12-26.) There is great variability in the size and shape of the platelets, but not any greater than is evidenced by Koch's and Kleine's figures. It is not a sufficient answer to the similarity I have shown to say that Kleine used defibrinated blood, and hence blood-platelets were not present in his solutions, for no one has demonstrated that the platelets are entirely removed by defibrination.

Since blood-platelets in various culture media and in the stomach of the tick always develop flagella, move about, and manifest such a marked resemblance in form, size, and structure to *Babesia* and the Leishman-Donovan bodies, investigators must furnish criteria to differentiate between the flagellated platelets and the parasites. Until they have established their position by experiments on normal blood, the correctness of their results can be accepted only with some reserve.

It is rather striking that Rogers and Kinoshita did not find in their sodium-citrate cultures the various forms that I have described,

since such forms are so easily observed in these cultures, and are apparently always present. It is also strange that Koch did not find forms with single, long, vibratile flagella. In my cultures and in the sheep tick these forms were numerous. Kleine, however, figures some with single, thick, and apparently rigid flagella.

It is not my intention to deny the development of a flagellate stage of *Babesia* in cultures and in insect hosts, but merely to call attention to the fact that in cultures of normal blood one can find forms similar, if not identical, to those ascribed to *Babesia* by some of the foremost investigators of this parasite. This similarity may aid in explaining the contradictory results of these investigators.

The evidence which I have presented shows that neither are motion and flagellation exclusive characters of parasites nor will they differentiate them from blood-platelets. Each student will have to determine experimentally how to distinguish the two classes of structures.

This study has been only incidental to another investigation and, therefore, by no means exhaustive. I am indebted to Dr. Henry B. Ward, of the University of Nebraska, for his direction of the investigation.

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#### EXPLANATION OF PLATE 1.

All figures are magnified approximately 2,800 diameters.

FIG. 1.—Normal blood-platelet from culture of human blood.

FIGS. 3 and 3.—Normal blood-platelets from a 3-hour citrate culture of sheep's blood.

FIG. 4.—Bipolar form from a 24-hour citrate culture of sheep's blood.

FIGS. 5-12.—Pear-shaped platelets with a single flagellum at the pointed end. Figs. 5, 6, and 7 from an 18-hour normal-salt-solution culture of sheep's blood. Figs. 8, 10, and 11 from a 24-hour citrate culture of sheep's blood. Figs. 9 and 12 from a 48-hour citrate culture of human blood.

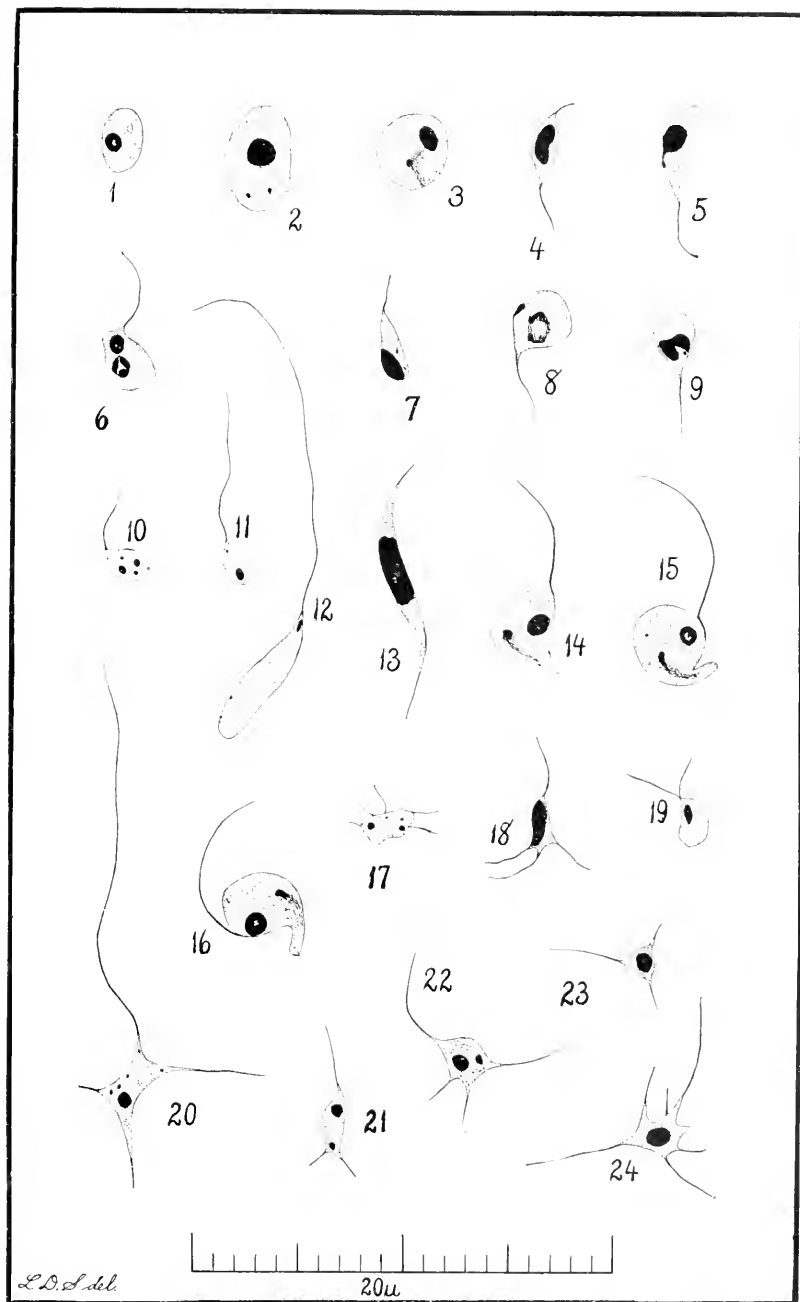
FIG. 13.—Biflagellated form from a 24-hour citrate and agar-agar culture of sheep's blood.

FIGS. 14-16.—Possibly monads which were accidentally introduced. Taken from a 24-hour citrate and agar-agar culture of sheep's blood.

FIGS. 17-24.—Blood-platelets resembling Koch's and Kleine's figures. Fig. 17 from a 27-hour citrate culture of sheep's blood. Figs. 18, 20, and 24 from a 24-hour citrate culture of human blood. Fig. 19 from an 18-hour-normal-salt-solution culture of sheep's blood. Figs. 21-23 from a 24-hour citrate and agar-agar culture of sheep's blood.



PLATE I.





## QUALITATIVE CHANGES IN THE THIRD SERUM COMPONENT.\*†

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IN work begun about three years ago for the purpose of testing the application of certain physico-chemical laws to hemolytic serum, a number of apparently paradoxical results were observed.<sup>1</sup> In attempting to determine the cause of these seeming paradoxes, the discovery was made that direct analytical methods are not applicable to many quantitative serum determinations.<sup>2</sup>

The application of quantitative methods is one of the most fundamental problems in any field of biological chemistry. It is the first problem that should be solved, before quantitative work is begun in that field. Failure to solve this problem may lead to experimental error and to erroneous conclusions. Work was therefore undertaken to determine why ordinary methods of analysis are not applicable to the phenomena in question, and, if possible, to find an indirect method applicable to them.

The problem immediately at hand was the measurement of the absorption of hemolytic amboceptor by blood corpuscles. Present analytical methods are not applicable to this problem, because the heated hemolytic serum, used for amboceptor measurements, is so changed by contact with corpuscles, that duplicate titrations do not agree.<sup>3</sup>

It was found that one of the reasons for this non-agreement of duplicate analyses was the probable change in the relative amounts of amboceptor and third component in such serum during exposure.<sup>4</sup> But the changes produced in a serum by altering the relative amounts

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<sup>1</sup> *Jour. Infect. Dis.*, 1905, 2, p. 490; *Centralbl. J. Bakt.*, 1906, 40, p. 382.

<sup>2</sup> *Jour. Infect. Dis.*, 1905, 2, p. 493; *Jour. Biolog. Chem.*, 1906, 1, p. 213; *Centralbl. J. Bakt.*, 1906, 40, p. 386; *Trans. Chic. Path. Soc.*, 1905, 6, p. 310.

<sup>3</sup> *Jour. Infect. Dis.*, 1908, 1, p. 67.

<sup>4</sup> *Jour. Infect. Dis.*, 1906, 3, p. 648.

of these two substances artificially, was in no case as pronounced as the changes observed in the same serum after exposure. It was therefore suspected that, in addition to such possible quantitative changes,

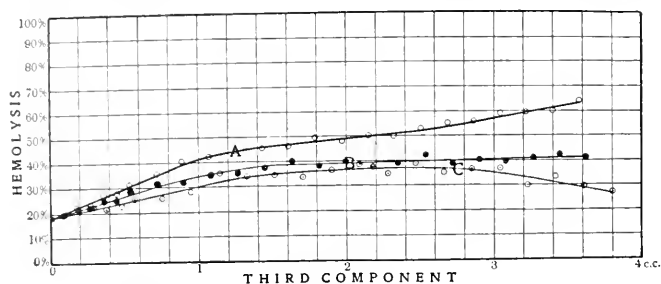


FIG. 1.—CHANGES IN THIRD COMPONENT DUE TO EXPOSURE TO CORPUSCLES.

A curve showing the changes in hemolytic power produced by increasing amounts of third component (heated normal goat serum) when added to a constant amount of hemolytic goat serum. *B* and *C* = curves showing changes produced by the same third component after contact with sheep corpuscles. The number of corpuscles used for curve *C* was greater than that for curve *B*. The constant amount of hemolytic serum used in the experiment was capable, in itself, of producing 18 per cent hemolysis. Curves *B* and *C* show a decrease in auxilic power, due to exposure to corpuscles.

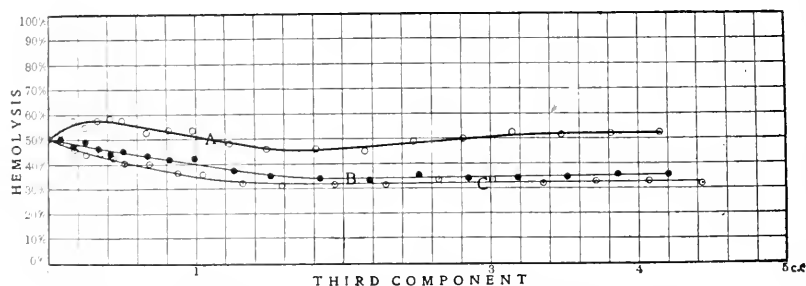
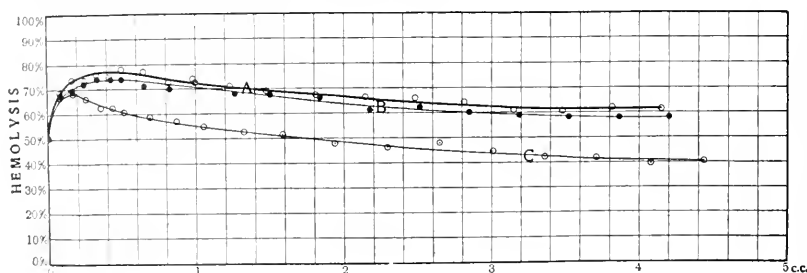


FIG. 2.—CHANGES IN THIRD COMPONENT DUE TO EXPOSURE TO CORPUSCLES.

*A* = unexposed third component; *B* and *C* = exposed third component. Curves otherwise similar to those of Fig. 1. Curve *C*, of the upper set, shows the auxilic power of unexposed third component replaced by an antilytic power. Curve *C* of the lower set shows a normal antilytic power increased after exposure.

there were changes of a qualitative nature as well. Can such changes be demonstrated?

In order to get a clew as to the nature of such possible changes, a study was made of the effect of exposing third serum component to sheep corpuscles. To do this, accurately measured quantities of third component<sup>1</sup> were allowed to stand in contact with carefully washed

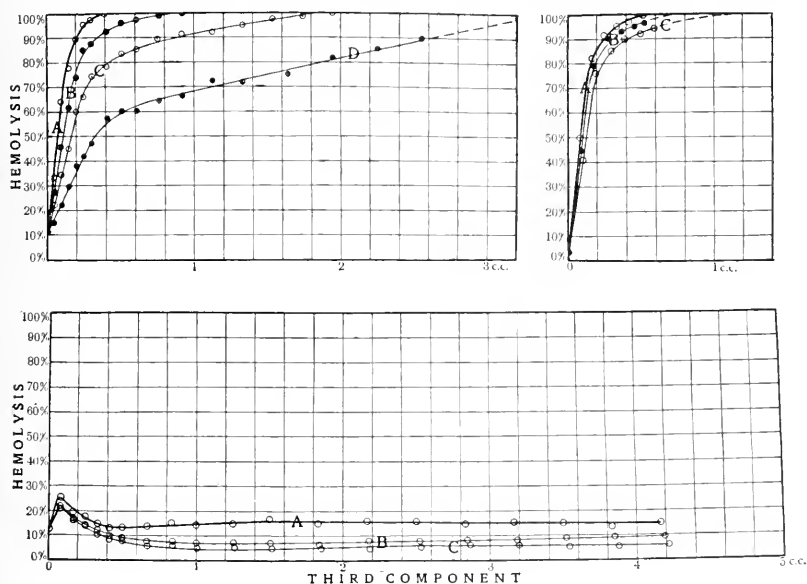


FIG. 3.—CHANGES IN THIRD COMPONENT DUE TO EXPOSURE TO CORPUSCLES.

A=unexposed third component; B, C, and D=exposed third component. Curves otherwise similar to those of Figs. 1 and 2.

sheep corpuscles, under the same conditions and for the same length of time as in the original amboceptor experiments.<sup>2</sup> The third component was then freed from the corpuscles by centrifugation, and its effect on hemolysis compared with that of the original component,<sup>3</sup> kept under identical conditions, except for the contact with corpuscles. Data, so obtained, are shown graphically in Figs. 1, 2, 3, and 4.

From these figures it is seen that exposure to corpuscles in all cases produces changes in the third component; that in every instance a

<sup>1</sup> Heated normal serum, containing neither amboceptor nor complement.

<sup>2</sup> For material and technic, see *Jour. Infect. Dis.*, 1905, 2, p. 461.

<sup>3</sup> For the action of the third component, see *Jour. Infect. Dis.*, 1906, 3, p. 647.

third component that is originally auxilytic (hemolysis-increasing) has its auxilytic power decreased by such exposure, or even replaced by an antilytic power; and that a third component that is originally antilytic has its antilytic powers increased.

Three hypotheses can be put forward to account for this change: First, that certain hemolytically active substances are absorbed from

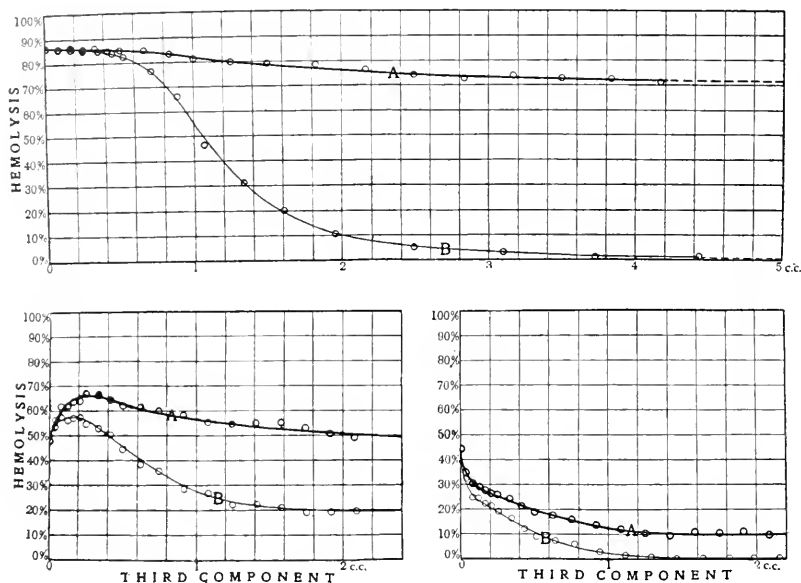


FIG. 4.—CHANGES IN THIRD COMPONENT DUE TO EXPOSURE TO CORPUSCLES.

A = unexposed third component; B = exposed third component. Curves otherwise similar to those of Figs. 1, 2, and 3.

the third component, by the corpuscles, during such exposure. Second, that certain hemolytically active substances are given off into the third component, from the corpuscles, during the exposure. And, third, that the corpuscles produce, independent of such absorption or giving off of products, chemical changes in the third component, presumably by the action of the enzymes they contain. Attempt was made to test these three hypotheses.

In order to determine whether or not active substances are absorbed by the corpuscles from the third component carefully washed corpuscles were exposed to third component, under conditions identical

with those of the amboceptor absorption experiments above, were then freed from the third component by centrifugation, repeatedly washed in salt solution, and their susceptibility to hemolysis compared with that of unexposed corpuscles, prepared, kept, and washed under identical conditions, except for the contact with third component.

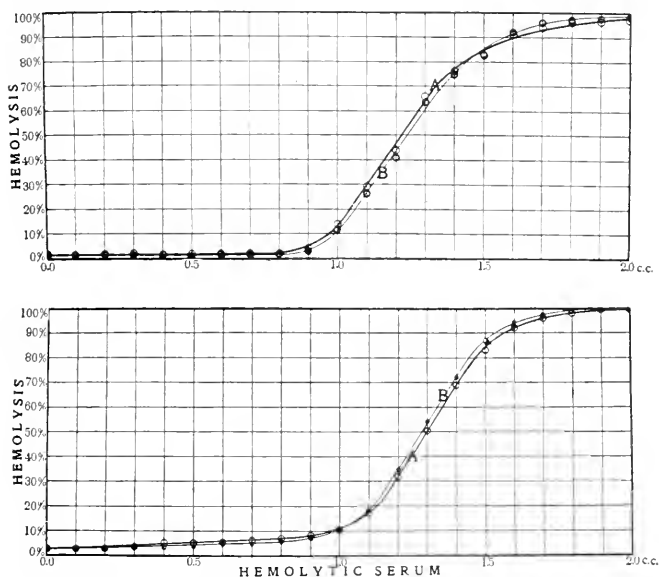


FIG. 5.—HEMOLYTIC SUSCEPTIBILITY OF CORPUSCLES AFTER EXPOSURE TO THIRD COMPONENT.

Each curve shows the percentages of hemoglobin liberated from a constant number of corpuscles, by increasing amounts of hemolytic serum. *A*=curves with unexposed corpuscles; *B*=curves with corpuscles after exposure to third component. The *B* curves show no change in hemolytic susceptibility after such exposure, within the limits of the experimental error. In the upper experiment the corpuscles were exposed to a powerfully auxilytic third component; in the lower experiment, to a third component practically inert.

The data from four such comparisons are given graphically in Figs. 5 and 6.

In the first two comparisons (Fig. 5), the corpuscles were found unchanged in hemolytic susceptibility, after exposure to third component, within the limits of the experimental error. In the second two comparisons (Fig. 6), changes were observed in hemolytic susceptibility, but in each case these slight changes were the opposite of those that would have followed a retention of third component by the corpuscles.<sup>1</sup>

<sup>1</sup> The slight changes observed in these corpuscles will be the subject of further investigation.

We are therefore obliged to conclude that the hemolytically active substances of the third component are either not absorbed by corpuscles, or, if absorbed, are held in such loose chemical combination that

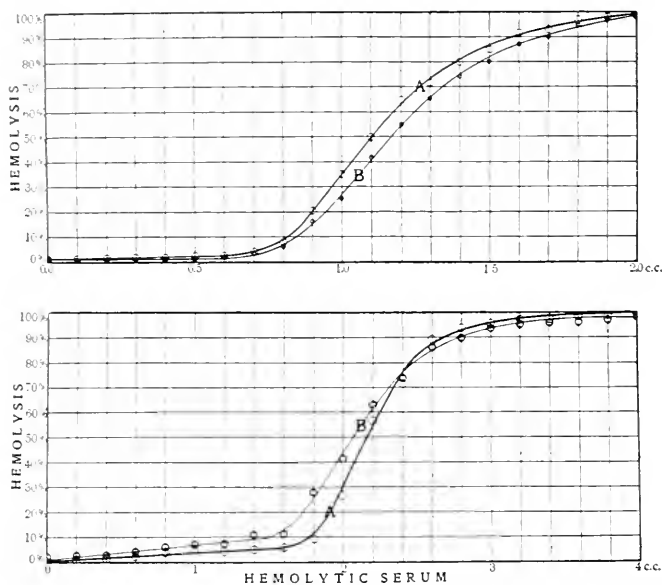


FIG. 6.—HEMOLYTIC SUSCEPTIBILITY OF CORPUSCLES AFTER EXPOSURE TO THIRD COMPONENT.

Curves, as in Fig. 5, showing changes in hemolytic susceptibility, after exposure to third component, that are not accounted for by the experimental error. In the upper experiment, the corpuscles were exposed to an auxilytic third component; in the lower experiment, to an antilytic third component. In each case the slight observed changes are the opposite of those that would have been expected from an absorption of the third component in question. The meaning of these changes is not clear, but they evidently are not changes due to third component absorption.

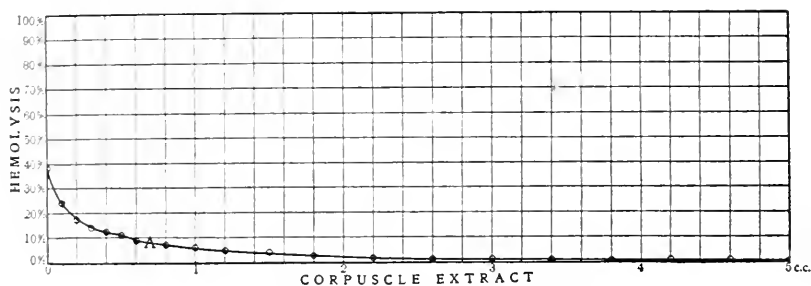


FIG. 7.—ANTILYTIC ACTION OF CORPUSCLE EXTRACT.

Curve showing changes in hemolytic power produced by adding increasing amounts of corpuscle extract (exposed salt solution) to a constant amount of hemolytic serum. The constant hemolytic serum used in this experiment was capable, in itself, of producing 38 per cent hemolysis.



they are completely removed by subsequent washing in salt solution. Absorption is experimentally undemonstrable.

In order to test the second hypothesis, that hemolytically active substances are given off from the corpuscles into the third component, washed corpuscles were exposed to physiological saline (0.85 per cent NaCl), under conditions identical with those of the amboceptor

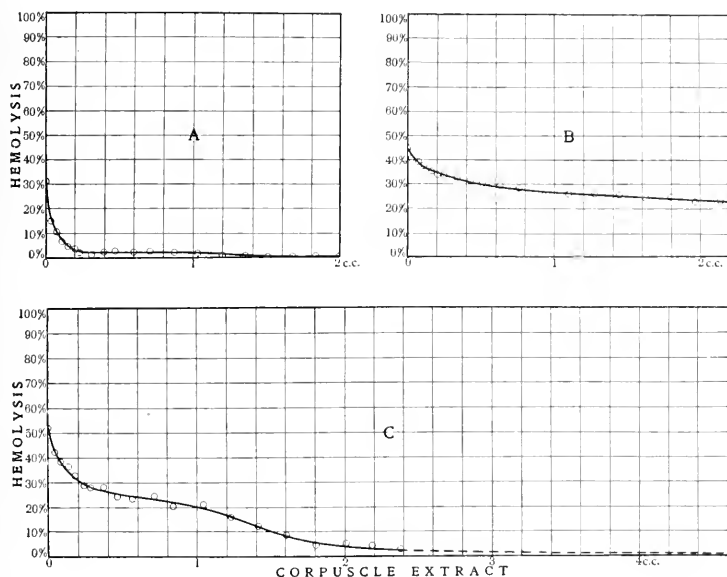


FIG. 8.—ANTILYTIC ACTION OF CORPUSCLE EXTRACT.

Curves, as in Fig. 7, showing changes in hemolytic power produced by adding increasing amounts of three different corpuscle extracts to different constant amounts of hemolytic serum.

absorption experiments above, the salt solution simply taking the place of the heated serum. The salt solution was then freed from corpuscles by centrifugation, and its effect on hemolysis tested.

It was found that this exposed salt solution (corpuscle extract) was in all cases powerfully antilytic. Such antilytic effects are shown graphically in Figs. 7, 8, and 9.

Corpuscles, therefore, give off a hemolytically active substance into salt solution. Is this substance also given off into third component? If so, and if the giving off of this substance is the only change brought about by corpuscles in exposed third component, it

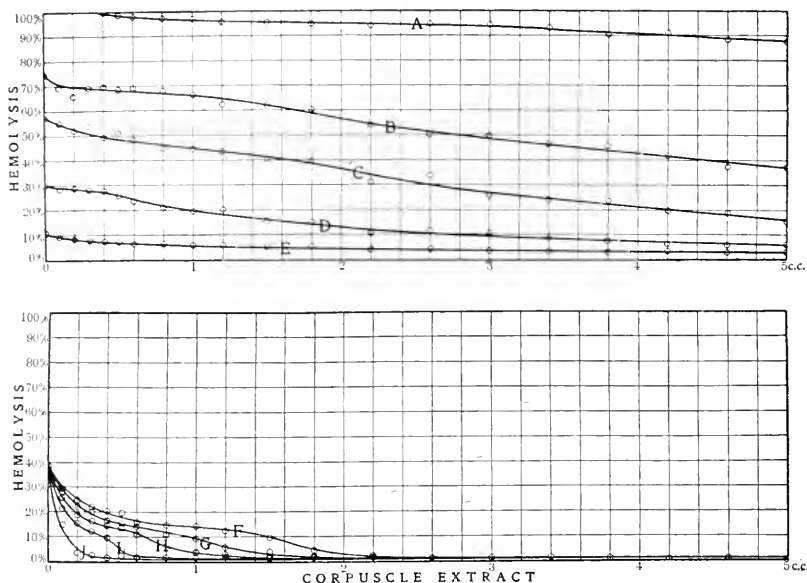


FIG. 9.—ANTILYTIC ACTION OF CORPUSCLE EXTRACT.

Curves, as in Figs. 7 and 8, showing changes in hemolytic power produced by adding increasing amounts of corpuscle extract, to constant amounts of hemolytic serum. *A, B, C, D, and E*=curves obtained with the same extract added to different constant amounts of the same hemolytic serum. *F, G, H, I, and J*=curves obtained with extracts from different numbers of the same corpuscles, added to a constant amount of the same hemolytic serum.

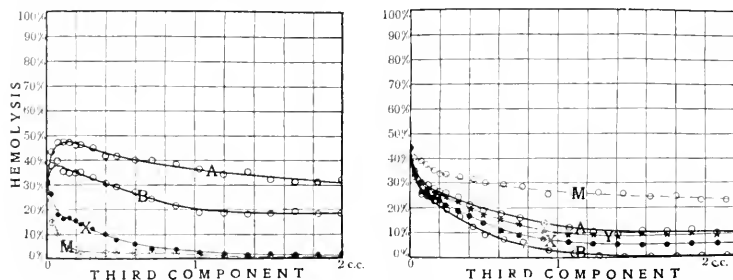


FIG. 10.—EFFECT OF CORPUSCLE EXTRACT ON THIRD COMPONENT.

Two sets of curves showing different effects on third component, of the addition of corpuscle extract. *A*=curve obtained with unexposed third component. *B*=curves with exposed third component. *M*=curves with corpuscle extract. This extract was in both cases obtained under the same experimental conditions, as those used in obtaining the exposed third component, salt solution simply taking place of the heated normal serum. *X*=curves obtained with a mixture of equal volumes of corpuscle extract (*M*) and unexposed third components. *Y*=curve obtained by adding a half-volume of corpuscle extract (*M*) to the unexposed third component.

In the first set of curves the addition of an equal volume of corpuscle extract produced a more marked change in the third component than the change produced in the same serum by exposure to corpuscles. In the second set of curves, the addition of an equal volume of extract produced a less marked change than such exposure.

should be possible to produce artificially the observed third component changes, by simply adding corpuscle extract to unexposed third component.

To test this possibility, various mixtures of unexposed third component and corpuscle extract were made, and the action of these mixtures compared with that of third component exposed to corpuscles. Four sets of data thus obtained are shown in Figs. 10, 11, and 12.

The essential curves from these figures have been grouped together in Fig. 13. From this figure it is seen that, in two of the experiments (I and III), the addition of

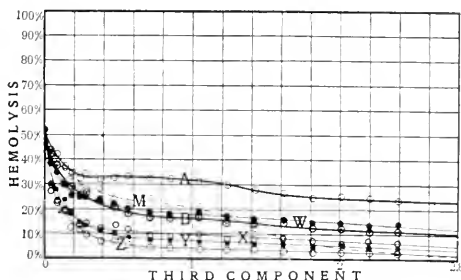


FIG. 11.—EFFECT OF CORPUSCLE EXTRACT ON THIRD COMPONENT.

Curves, as in Fig. 10, showing the effect on the third component of the addition of corpuscle extract. *A*=curve with unexposed third component. *B*=curve with exposed third component. *M*=curve with corpuscle extract. *W*=curve obtained by adding a half-volume of corpuscle extract (*M*) to unexposed third component. *X*=curve with the addition to an equal volume of extract. *Y*=curve with one and a half volumes of extract. *Z*=curve with twice the volume of extract. In this experiment the nearest approach to the exposed third-component curve was produced by the addition of a half-volume of extract (see Fig. 14).

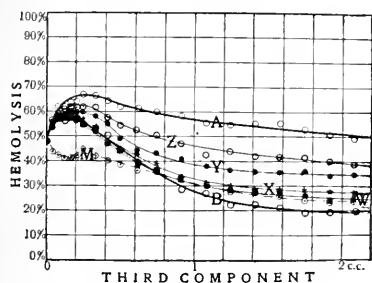


FIG. 12.—EFFECT OF CORPUSCLE EXTRACT ON THIRD COMPONENT.

Curves, as in Figs. 10 and 11, showing the effect on the third component of the addition of corpuscle extract. *A*, *B*, and *M*=curves with unexposed third component, exposed third component, and corpuscle extract, as before. *Z*=curve obtained by the addition of a quarter-volume of corpuscle extract. *Y*=curve with a half-volume. *X*=curve with a three-quarter volume. *W*=curve with an equal volume. In this experiment, the nearest approach to the exposed serum is made by the addition of an equal volume of corpuscle extract.

an equal volume of corpuscle extract to unexposed third component, conferred on the component greater antilytic powers than that acquired by exposure to corpuscles. In a third experiment (II), the addition of an equal volume conferred less auxilytic powers; while in a fourth experiment (IV), it gave greater antilytic power when the component was tested in certain amounts, but less antilytic power when tested in other quantities.

The nearest artificial approximation to an exposed third component was obtained, in one of

the experiments (III), by adding a half-volume of corpuscle extract. The nearly coincident curves from this experiment are shown in Fig. 14.

The addition of corpuscle extract to third component, therefore, produces changes in the third component, approximating those produced by exposure to corpuscles, but not identical with them. If we

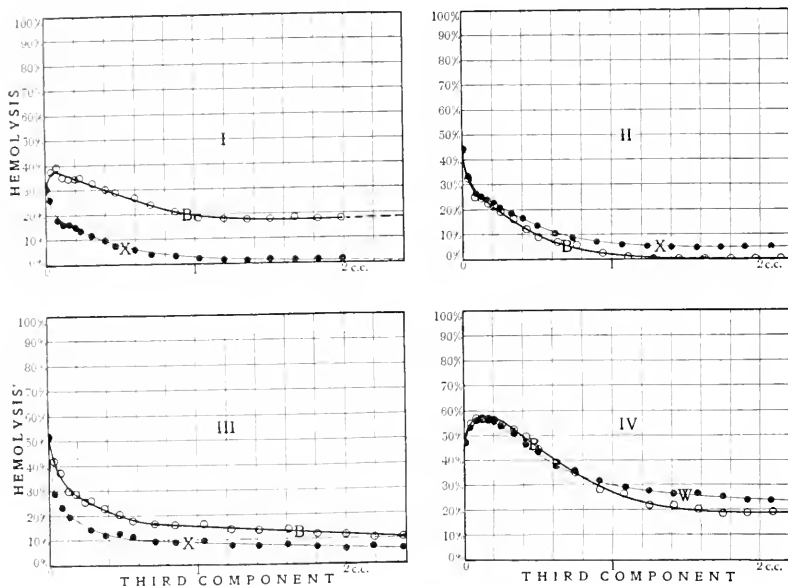


FIG. 13.—EFFECT OF CORPUSCLE EXTRACT ON THIRD COMPONENT.

Curves obtained with the addition of equal volumes of corpuscle extract, brought together from FIGS. 10, 11, and 12, above.

assume that the changes produced in exposed third component are mainly due to the giving off of corpuscle products into that component, it will be necessary to assume that the amount of these products so given off is influenced by the nature of the third component used; that with one third component there is given off an amount equal to that given off by the same corpuscles into physiological saline; that with another third component a much smaller amount is given off than in the corresponding experiment with physiological salt solution; while with a third experiment a much larger amount is given off.

Moreover, this hypothesis would still leave unaccounted-for certain

minor changes in the third component, indicated by the crossing of the approximating curves. What this change is, and whether or not it can be accounted for by a hypothetical digestion of third component by corpuscle enzymes, is still a matter of pure conjecture.

The qualitative changes produced in the third serum component by exposure to corpuscles are therefore such that they can neither be predicted nor reproduced artificially with sufficient accuracy for analytical purposes. This being the case, it does not seem possible at present to devise an indirect method of analysis by means of which the absorption of hemolytic amboceptor can be determined.

In the light of this conclusion, it would seem desirable to examine with care fundamental analytical data in other fields of serum pathology, to determine whether or not they rest on a sufficiently reliable foundation for the theoretical deductions now made from them.

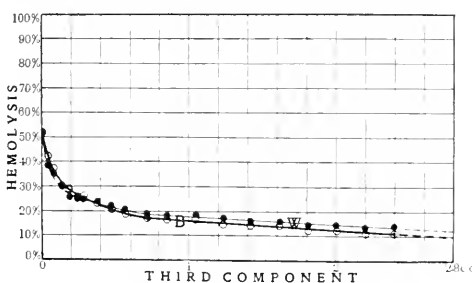


FIG. 14.—EFFECT OF CORPUSCLE EXTRACT ON THIRD COMPONENT.

The nearly coincident curves of Fig. 11.

#### SUMMARY.

1. Heated normal goat serum (pure third serum component) is altered in its chemical nature by exposure to washed sheep corpuscles. A third component that is originally auxilytic has its auxilytic powers decreased by such exposure, while a third component originally antilytic has its antilytic powers increased.

2. Absorption of the third serum component by the exposed sheep corpuscles is not demonstrable.

3. Washed sheep corpuscles give off into exposed physiological saline a powerful antilytic substance.

4. The addition of this antilytic corpuscle extract to unexposed third component produces approximately the same changes in the third component as those produced by exposure to corpuscles. The amount of extract necessary to produce the desired change, however,

varies with different sera. With one third component it may be the same amount as that given off by duplicate corpuscles, under identical experimental conditions, into physiological saline. With another third component, it may be a much smaller amount, and with a third serum, a much larger amount.

5. If we assume that the changes taking place in exposed third component are mainly due to the addition of corpuscle products, it will be necessary to assume certain factors in the third component that influence the amount of these products so acquired. In the presence of one third component, the giving off of these products by the corpuscles must be assumed to be stimulated; with another third component, retarded; while with a third it is not influenced.

6. Moreover, distinct differences between experimental curves indicate, that, in addition to the giving off of corpuscle products into third component, there must be assumed certain minor secondary changes in the third component, the nature of which is still a matter of conjecture.

7. The changes in exposed third component are so complex, that an indirect method of determining the amboceptor absorption by sheep corpuscles seems at present impossible.

## FACTORS IN HEMOLYSIS.\*†

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DURING an investigation in hemolysis, extending over about three years, a number of unsuspected phenomena have been encountered. The discovery of these phenomena has modified the original comparatively simple concept of hemolytic serum, so that in place of the two components at first regarded as the only active substances in such serum, at least a dozen components are now recognized.<sup>1</sup> Some of these components have been found under certain conditions to be beyond experimental control, and unrecognized, to introduce serious error in hemolytic investigation.

The experimental work, of which this paper is in a way a summary, was done with goat serum immunized against sheep corpuscles. The substances herein enumerated, therefore, must be regarded as found only in such serum. These substances are:

1. AMBOCEPTOR. A specific, thermostable substance, or group of substances, formed in goat serum in response to repeated injections of washed sheep corpuscles. This substance is not destroyed by heating to 60° C. for several hours, or by standing at ordinary temperatures for months. Amboceptor is a necessary factor for the hemolysis of sheep corpuscles by goat serum; hemolysis not taking place in its absence, regardless of the number or the amount of the other serum components present.

The action of amboceptor is not understood. The current belief that it is absorbed by corpuscles rests on doubtful experimental grounds, as the quantitative analysis of an amboceptor containing goat serum that has once been in contact with sheep corpuscles is at present out of the question.<sup>2</sup> One can only say that contact with amboceptor in some way renders sheep corpuscles susceptible to

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<sup>1</sup> For techn., see *Jour. Infect. Dis.*, 1905, 2, p. 461.

<sup>2</sup> *Jour. Infect. Dis.*, 1907 4, p. 219.

hemolysis by certain other components of goat serum, which to unexposed corpuscles are non-hemolytic. No trace of amboceptor is demonstrable in normal goat serum.

2. COMPLEMENT. A non-specific, thermolabile substance, or group of substances, found in normal goat serum. Whether or not the amount or nature of the complement is changed, as a result of repeated injections of sheep corpuscles, is not known. Goat complement is apparently completely destroyed by heating to 50° C. for 60 minutes, to 55° C. for 15 minutes, or to 60° C. for 5 minutes.<sup>1</sup>

Complement is a necessary factor for the hemolysis of sheep corpuscles by goat serum; hemolysis not taking place in its absence, regardless of the number and amount of the other serum components present. In itself complement is non-hemolytic, requiring for its action the presence of a certain amount of amboceptor. Sheep corpuscles, however, that have been exposed to goat amboceptor are readily hemolyzed by complement alone. The nature and the method of action of complement are not understood.

3. THIRD COMPONENT. A collective name applied to the other constituents of goat serum. The nature of these constituents is impossible of experimental study in unheated sera, due to the presence of the hemolytically more active complement. A study of normal goat serum in which the complement has been destroyed by heat, however, shows that the third component then apparently consists of at least three hemolytically active substances, or groups of substances. These are: (1) an antilysin, or hemolysis-inhibiting substance, and (2) two auxilysins, or hemolysis-increasing substances.<sup>2</sup>

The nature of these substances is not understood. Whether they are independent substances or not, and whether or not they are derivatives of complement, that is, "complementoids," have not been determined. The amounts of these substances apparently differ considerably in sera from different goats, and in the serum of the same goat at different times and under different experimental conditions. Their chemical composition, however, is apparently the same in different sera.

The third component in itself is non-hemolytic, and is incapable

<sup>1</sup> *Trans. Chi. Path. Soc.*, 1906, 6, pp. 425-27.

<sup>2</sup> *Jour. Infect. Dis.*, 1906, 3, pp. 225, 226, 647.



of producing hemolysis in the presence of pure amboceptor or pure complement. It is believed that many current errors in hemolytic theory are due to failure to take into account the presence of, and variations in, this component.

4. CORPUSCLE EXTRACT. Washed sheep corpuscles probably give off into the surrounding medium at least three substances capable of modifying hemolysis. These are: (1) traces of sheep complement, whose action is apparently identical with that of goat complement, (2) traces of sheep third component, whose action is believed to be similar to that of goat third component, and (3) certain substances believed to be products of corpuscle autolysis.<sup>1</sup>

The principal active component in these autolytic products is a powerful, antilytic, thermostable substance, whose nature is not understood. It is the presence of this substance that renders the quantitative analysis of amboceptor-containing goat serum impossible, after the serum has been exposed to sheep corpuscles. Whether or not similar substances are given off into goat serum from its own corpuscles, has not been determined.

5. HEMAGGLUTININ. A specific, thermostable substance, or group of substances, formed in goat serum in response to repeated injections of sheep corpuscles. The amount of this substance varies greatly in different sera. One serum may produce complete hemagglutination in a few minutes, while another serum of apparently equal hemolytic power may be practically without agglutinating action.

The nature of this hemagglutinin is not understood. Whether or not it bears any chemical relation to amboceptor, is unknown. Its effect on hemolysis is believed to be to decrease lysis, by clumping corpuscles and thus removing them from the action of amboceptor and complement. No trace of hemagglutinin is found in normal goat serum.

6. ACCESSORY HEMAGGLUTININS. At least two substances, or groups of substances, have been encountered that modify the rapidity or the completeness of the agglutinating process. These are: (1) an auxagglutinin,<sup>2</sup> or agglutination-increasing substance in the third component, and (2) an antiagglutinin, or agglutination-decreasing substance in corpuscle extract. The nature of these substances is not

<sup>1</sup> *Jour. Infect. Dis.*, 1907, 4, p. 219.

<sup>2</sup> *Ibid.*, 1906, 3, p. 227.

understood, and their relation to the other active substances in third component and corpuscle extract is not known.

7. CORPUSCLE SUSCEPTIBILITY. Changes in the susceptibility of washed sheep corpuscles, both to hemolysis and to hemagglutination, have been observed. The nature of these changes is not understood, and their importance in hemolytic work is undetermined. It is possible, however, that they introduce error in certain hemolytic investigations.

8. INORGANIC SALTS, SPECIFIC GRAVITY, REACTION OF MEDIUM. Certain inorganic constituents of serum, especially the calcium salts, are known to be powerfully autolytic.<sup>1</sup> The influence of specific gravity on hemolysis is not accurately known, but it is possible that errors may arise from variations in it. The reaction of the medium, that is, its degree of acidity or alkalinity, is known to influence greatly its hemolytic action. The importance of these three factors, however, in hemolytic investigation, has not been adequately determined.

9. HEMOPSONIN. It is suggested by Dr. Hektoen that the corpuscles in a hemolytic tube might possibly become opsonized by the hemopsonins<sup>2</sup> of the immune goat serum, and that some of them might be taken up by the leucocytes of the blood suspension and protected from hemolysis. Numerous smears made from routine hemolytic tubes, however, show no sign of such phagocytosis.

In the routine technique, the corpuscles are 24 hours old. The above examination, therefore, does not exclude the possibility of phagocytosis playing a rôle in hemolytic experiments under a technic involving the use of fresher corpuscles. Such a rôle, however, must be practically negligible, as it is inconceivable that the leucocytes could take up more than a maximum of  $\frac{1}{4}$  per cent of the red corpuscles present, which would introduce a maximum error in the hemoglobin liberation less than the minimum difference in tint that can be detected by ordinary colorometric methods.

10. PRECIPITINS. It is further conceivable that specific precipitins may have an influence in certain hemolytic experiments. No trace of such precipitins, however, has been found in goat serum immunized against washed sheep corpuscles or against normal sheep serum.

<sup>1</sup> *Jour. Infec. Dis.*, 1904, 1, p. 379.

<sup>2</sup> *Ibid.*, 1906, 3, pp. 721

## SUMMARY

The substances above enumerated may be tabulated, as follows:

- |                       |                        |                             |                  |                              |
|-----------------------|------------------------|-----------------------------|------------------|------------------------------|
| I. NECESSARY FACTORS  | }                      | 1. Amboceptor               |                  |                              |
|                       |                        | 2. Complement               |                  |                              |
|                       |                        | 3. Third Component          | {                | (1) Antilysin                |
|                       |                        |                             |                  | (2) Auxilysin I              |
|                       |                        |                             | (3) Auxilysin II |                              |
| II. ACCESSORY FACTORS | }                      | 4. Corpuscle Extract        | {                | (1) Complement               |
|                       |                        |                             |                  | (2) Third Component          |
|                       |                        |                             |                  | (3) Autolytic Products       |
|                       |                        | 5. Hemagglutinin            |                  |                              |
|                       |                        | 6. Accessory Hem-           | {                | (1) Serum Auxagglutinin      |
|                       |                        | agglutinins                 |                  | (2) Corpuscle Antiagglutinin |
|                       |                        | 7. Corpuscle Susceptibility |                  |                              |
|                       | 8. Inorganic Salts     |                             |                  |                              |
|                       | 9. Specific Gravity    |                             |                  |                              |
|                       | 10. Reaction of Medium |                             |                  |                              |

In enumerating these components, no claim is made that they are all independent substances. Nothing is known of their chemical composition, nor of the relations existing between them. Their enumeration, however, is believed to be of value, as it indicates the kind of complexities that may be expected in other fields of serum pathology.

## THE SIGNIFICANCE OF LEUCOCYTES AND STREPTOCOCCI IN THE PRODUCTION OF A HIGH-GRADE MILK.\*

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AMONG the many unsettled problems of modern dairying is that of the significance of leucocytes and streptococci in milk. The literature on the question is varied and plentiful and there is a great divergence of the opinions of even the most careful workers and observers. Harris<sup>1</sup> closes his paper on the subject by saying "The time seems ripe for throwing open the whole question for discussion." Especially is this the case since certain municipalities, in view of the evidence in favor of the conclusion that the presence of numerous leucocytes and streptococci, either singly or together, denotes disease in the cow, have felt justified in excluding such milk from sale and in demanding that the producer remedy the matter if he desires to continue supplying the city trade.

That the presence of streptococci or pus in milk is sometimes the visible indication in the milk of disease in the cow injurious to the consumer, no fair-minded person will deny. That the presence of such elements is not always followed by injury to the average consumer is equally apparent. However, from the standpoint of our boards and commissions of health, upon whom rests, very largely, the responsibility of our enormous infantile death rate, there must be formulated regulations which will work the greatest good to the health and well-being of the community and the minimum of hardship to the minority compatible with the attainment of the purpose.

Any inspection of a city's milk supply is difficult, expensive, and annoying because of the ignorance and carelessness of the producer and his propensity for trying to deceive. An inspection involving laboratory work increases all the difficulties. However, an honest endeavor is being made by certain municipalities to carry out laboratory inspection in so far as streptococci and leucocytes are concerned,

\* Received for publication January 9, 1908.

<sup>1</sup> *Jour. Infect. Dis.*, 1907, Supplem. 3, p.

and it is reasonable to suppose that they consider the end to justify the means. They are making an effort, so far as their facilities will permit, to trace the milk to the dairy, and there confirm or deny the laboratory findings.

During 1905 the city of Boston, using the method of Slack for the detection and estimation of streptococci and pus in milk, reported 10.48 per cent of its milk supply thus contaminated. Pus alone was found in 5.01 per cent, pus and streptococci in 1.09 per cent, and streptococci alone in 4.37 per cent, 5,559 samples having been examined. This report expresses the opinion that pus or streptococci render milk unfit for human consumption and indicate disease in the cow. A list of 33 cases is given in which the conditions at the dairy warranted the conclusions reached by the laboratory.

The city of Philadelphia, in 1904, when such inspection was organized, reported pus in 4.2 per cent, pus and streptococci in 0.93 per cent, and streptococci alone in 7.19 per cent, 9,243 samples of milk having been examined; 324 positive findings in the laboratory were confirmed by conditions at the dairies; in 48 cases veterinary inspection failed to show why the laboratory found pus or streptococci in the milk. In 1905, when the work of laboratory inspection was better organized, 9.99 per cent were reported for pus, 2.01 per cent for pus and streptococci, and 11.35 per cent for streptococci alone; 624 of these condemnations were followed to the dairy. The results are given in Table I.

TABLE I.

A COMPARISON OF THE REPORTS OF THE VETERINARIANS EXAMINING THE HERDS WITH THE FINDINGS OF THE LABORATORY BECAUSE OF WHICH THE INSPECTION WAS MADE.\*

VETERINARIAN REPORTED	THE LABORATORY PREVIOUSLY FOUND				
	Pus	Streptococci	Pus and Streptococci	Blood and Streptococci	Blood and Pus
Inflammation (mammitis, garget, etc.)	107	145	48	1	...
Milking too close to parturition.....	22	43	5	...	...
Milking for too long a period.....	5	8	1	...	...
Cow pox.....	2	1	1	...	...
Out of condition.....	1	11	...	...	...
Wounds of udder.....	7	9	1	...	...
Ropy milk.....	1	3	...	...	...
Cows were excluded but no reason was given.....	18	38	9	...	...
No disease observed when the examination was made.....	84 (37%)	64 (19%)	13 (16%)	...	1
	222	322	78	1	1

\* Report of Bacteriological Laboratory of the Bureau of Health of Philadelphia, 1906.

The returns from these dairy examinations, for both 1904 and 1905, show a certain number stating that "no disease was observed when the examination of the herd was made." Such cases are, for the two years mentioned, about 20 per cent of the whole number investigated. It must not be forgotten, however, that the notification to the farmer was made by post; that in many of the country districts the mail is irregularly delivered or called for, so that delays are always likely to occur on this account. The farmer may also defer calling the veterinarian to make an examination, and it may be a matter not only of days but sometimes of a week or two before the veterinarian reaches the farm to examine the cows in question. Such being the case, it is more than likely that the conditions which prevailed when the milk was examined had either remedied themselves or become so noticeable that the farmer himself was compelled to take them into account. It would seem, therefore, that depending, as the city must, on a tardy, irregular, or incomplete examination of the herd, the percentage of agreement between its laboratory findings and the reported examinations show rather an unusual accord.

The method which has been used for the detection of streptococci and of the number of leucocytes present in the milk is the centrifuge method of Stewart. For an accurate enumeration of the number of cells in milk this method will not serve. For the routine examination of a large number of milk samples, however, when the exact count of the leucocytes is not required, but rather a knowledge sufficient to indicate the fitness of that particular milk for food, we believe the method to be eminently satisfactory. Without doubt the number of leucocytes obtained by this method is lower than, for example, that by the Doane-Buckley method, but up to the present time we have not been able to fix absolutely a numerical leucocyte standard for the indication of disease in the cow; and the diagnosis of such from the sediment of the milk must, until this value is fixed, depend very largely upon the familiarity of the observer with the particular method of enumeration which he is using, and the condition in the animal which, by experience, he finds to parallel his microscopic results.

It is doubtful, too, when the examination of a large milk supply, as for a city, or the examination of the individual cows of a large herd,

is under consideration, whether the extra time consumed by the requirements of one of the more accurate numerical methods is not more than overbalanced by the additional number of samples which such a method as Stewart's enables one to investigate. The use of any of the established methods for the study of leucocytes in milk furnishes the person who is examining many samples from many sources with information which is not confined solely to the number of cells per cubic centimeter.

The often-quoted statement of Savage<sup>1</sup> that he "cannot differentiate between a leucocyte and a pus cell" is undoubtedly true so far as the comparatively few isolated cells of a clean milk are concerned. However, it does seem possible, in a large percentage of cases, to determine, by considering the relative number of the various kinds of leucocytes going to make up the cell content of the milk, whether they are polymorphonuclear, large or small mononuclear, eosinophiles, etc.; and by their staining and appearance, whether they are degenerated or in good condition morphologically, loose lying cells or collected into masses with evidences of fibrin; and from such observations it seems possible to determine whether the condition which produces them is likely to be pathological or normal.

It may be that streptococci in milk, from animals showing no clinical signs of mastitis, are not pathogenic to laboratory animals. Kruse,<sup>2</sup> Hölling,<sup>3</sup> Heinemann,<sup>4</sup> and others have put forth the view that the streptococcus found in milk, which they term *Strept. lacticus*, is a constant inhabitant of the cow's udder and is, in reality, the lactic-acid-forming organism of milk. Heinemann, in a later paper,<sup>5</sup> states that this organism, both morphologically, culturally, and in pathogenic property, is closely related to *Strept. pyogenes*; that, in doses of 2 c.c. of 24-hour old broth culture, given intravenously, it is fatal to rabbits, and passed subcutaneously through several animals, its virulence increases so that the above amount is fatal in subcutaneous injections. Petruschky and Kriebel,<sup>6</sup> however, attribute much of the summer infantile diarrhea to these organisms in milk. Escherich<sup>7</sup> has made a lengthy study of streptococcic enteritis in children, finding a greater number of the organisms in the intestines of infants fed on cow's milk than in those of breast-fed children. Lammeris and von Harrevelt<sup>8</sup> report serious illness traced to the *sterilized* milk of a herd in which one cow had had mastitis. She had been clinically well for some days and it was not supposed that her

<sup>1</sup> Jour. Hyg., 1906, 6, p. 123.

<sup>2</sup> Centralbl. f. Bakt. Abt. I, 1903, 34, p. 737.

<sup>4</sup> Jour. Infect. Dis., 1906, 3, p. 173.

<sup>3</sup> Dissertation, Bonn, 1904.

<sup>5</sup> Ibid., 1907, 4, p. 87.

<sup>6</sup> Die Ursachen der Sommersterblichkeit der Säuglinge u. die Möglichkeit ihrer Verhütung, Leipzig, 1904.

<sup>7</sup> Jahrb. f. Kinderheilk., 1899, 49, p. 137.

<sup>8</sup> Ztschr. f. Fleisch- u. Milchhyg., 1901, 11, p. 114.

milk was still infected. Jacobsen<sup>1</sup> traced an infection among adults to a herd where four cows showed streptococci in their milk, but the clinical signs were too slight to be observed by the milkers.

It has been observed by one of us in the examination of many samples of milk from various sources made because the children fed on them were either acutely ill or did not thrive, that in a very great majority of the cases a streptococcus was present. Many times the milk was, otherwise, above criticism. Frequently there has been an opportunity to examine each individual cow furnishing the milk, and when the animal giving streptococci was located and excluded the trouble ceased.

We have no positive method for determining before the milk is consumed whether the organisms which morphologically are streptococci are or are not virulent to all animals of greater and of less susceptibility. Therefore, in our present state of knowledge a milk, to be above reproach, must be free from streptococci. The physicians, parents, or nurses sufficiently progressive to have such laboratory examinations made, usually select a source of supply above the average; hence it is an exception to find a cow in such herds having any clinical signs of udder inflammation.

In the past few years four cases have been observed in which the human mammary gland, without any signs whatever of inflammation, yielded, in the milk, pus and streptococci. Three of the infants fed from these breasts were desperately ill when the milk was brought for examination. The other, an infant only two weeks old, was losing weight and had green stools. All the children recovered when taken off the breast.

A large number of leucocytes in milk may not be harmful, but their presence does, sometimes, stand for definite pathological conditions and, therefore, milk with numerous leucocytes<sup>2</sup> may cause digestive disturbances or such obscure disorders as now baffle the pediatrician.

Both pus and streptococci in milk may be detected in the laboratory in a brief space of time and it has seemed to us possible to make the laboratory of very practical service to the dairy in this matter, and also to accumulate some data which cannot be obtained where the lack of harmony is so pronounced as it is between the health boards and the usual dairymen.

<sup>1</sup> *Ztschr. f. Fleisch- u. Milchhyg.*, 1901, 11, p. 114.



The examination, yearly, of approximately 15,000 samples of milk by the municipal laboratories of Philadelphia and Boston, from all sorts of sources, must furnish results which are entitled to consideration. That such work leaves much to be desired because of the imperfect facilities which now prevail for the correlation of the conditions at the dairy and the results obtained in the laboratory, is equally true. It has been deemed worth while, therefore, to set forth some of the observations made, so far as pus and streptococci are concerned, on a herd which is being maintained for the production, commercially, of a milk of the highest possible quality, around which so many safeguards shall be thrown that it may be used with impunity, unheated, for the most susceptible infant or delicate invalid.

To such an end a dairy consisting of about 70 milking cows sends to the laboratory daily the milk of such a number of cows as will enable the laboratory to examine each individual in the herd weekly. It also sends a sample of mixed milk of the herd. The samples are examined by the Stewart method and the findings communicated promptly to the dairy. If an animal is reported as having streptococci in her milk she is excluded from the herd until the organisms disappear. Streptococci are very seldom found in the cows of this herd, and in almost every instance a reason for their presence has been forthcoming when conditions were analyzed. When pus is reported the cow is examined clinically, and if no evidences of disease are present, her record is looked up with the result, very frequently, that the cause of the pus is made clear.

This system was started about two years ago and so far has been most successful. A study of the records show that there is in the majority of cases a close connection between large numbers of leucocytes in milk and the physical condition of the cow both in relation to and independently of udder inflammation.

We have found, for example, that the close of the period of lactation is very sharply indicated. It is a textbook assertion that pus makes its appearance if a cow is milked too long. That such a condition is recognized widely is indicated by the many reports sent to the Board of Health of Philadelphia stating that, when pus was found, the milking had been too long continued. It was not surprising, therefore, when making consecutive observations on a care-

fully watched herd, to find that a cow, which throughout her whole period of lactation had given a milk low in leucocytes, should suddenly develop pus, and that such a development indicated the close of her milking period. In almost 75 per cent of the herd, perfectly healthy, clean cows show pus at the close of the milking period and a coincident chemical analysis indicates that the percentage of butter fat is from 0.5 to 1.5 per cent above the normal for that particular individual. Such a condition is interpreted as indicating the termination of lactation, and accordingly the cow is at once dried off with, apparently, advantage to both herself and her calf.

Of some significance also, in the study of increased leucocytes in the milk of clinically perfect cows is the fact that, from the herd which we are watching, 19 cows have been sold during the year 1906, not for specific defects, but because in most cases they were unsatisfactory milkers and were not commercially profitable owing to their comparatively small yield. Of these 19 animals, 15 showed pus in a large proportion of examinations, but had no physical signs of udder trouble. One cow had positive findings seven times between April 1 and April 25, when she was sold; another had eight positive reports within the three months preceding her sale; another showed pus five times in about 12 weeks, and so on.

It is possible that the frequency of excessive numbers of leucocytes in cows of mixed blood which fall below a paying milk yield is dependent to a certain extent upon the ration fed. This must of necessity be as high as is compatible with the health of the herd. It is varied as much as possible and contains always grass pasture or ensilage (the latter being substituted for grass from October to May), and such other foods as alfalfa, clover hay, malt sprouts, sucrene feed, beet pulp, buckwheat middlings, dried brewer's grains, and gluten. There is fed, however, very little gluten. It has seemed to us possible that some cows might respond to the high feeding with increased leucocytosis, rather than with increased milk yield, and this point is one which we hope later on to investigate practically.

If a chemotaxis of this character occurs in cows which are constitutionally small milkers, a study of cows which are, from the standpoint of the dairyman, admirably adapted to the business for which he keeps them, is of interest as affording light on the reverse of

the proposition. Accordingly, from their behavior in the barn, their milk yield, and general fitness, the best 10 cows in the herd were selected for study. The laboratory records of these cows showed that in their milk the presence of pus was scarcely ever noted except at the close of lactation or when they were suffering from cow pox; that in not a single one of the 10 have streptococci ever been observed, and some of these animals have been in the herd for three years. Not one of the 10 is a Jersey; only one has any quantity of Guernsey blood and her cross is Durham; and others are Ayrshire, Holstein, and Durham and are, for the most part, thoroughbreds. So striking has been the cleanliness of the milk of the above breeds as compared with that of Jerseys and Guernseys, when kept under just the same conditions, that we propose making a more careful study of this phase of the production of a sanitary milk.

In certain cases we have observed many degenerated leucocytes in milk when there has been a constitutional disturbance entirely foreign to the udder. Such, for instance, was noted in a clean cow which caught a heavy cold accompanied by congestion of the lungs. It lasted for about a month, during which time the milk showed pus. After the cow regained her health, the pus ceased. She never showed the slightest indication, clinically, of udder inflammation.

Probably no greater calamity can befall a dairy herd than the appearance among them of an infectious mammitis, unless it be a tubercular infection. It would seem to be fairly well established that not only can the entire output of milk be contaminated by one animal, but she may be responsible for the illness of the whole herd. We believe that a strict laboratory surveillance of each cow, with a close watch clinically, greatly lessens the likelihood of the spread of such an infection. Pus and streptococci persist in milk in an attack of mastitis after all physical signs have disappeared; and one or the other, or both, appear before clinical signs are manifest. It has been our observation, also, that when a cow has once shown evidences of udder inflammation and continues to exhibit a tendency to a high leucocyte count, she is apt to acquire a streptococcus showing. Such cows are always a source of worry, if of nothing else. For example, a cow, No. 57, shortly after calving, showed pus and a staphylococcus in her milk. This condition cleared up within a week, but excessive

leucocytes from time to time persisted with, finally, three consecutive findings of streptococci associated with pus and evidences of udder inflammation. The cow was not very profitable, and to prevent a possible infecting of the herd, she was sold.

This is a condition which has been noted a number of times. Increased leucocytes appear and, without any clinical evidence of disease, persist for weeks. Then streptococci are noticed. With such a history the cow is either quarantined or sent to the butcher, generally the latter, since it has been deemed better to sacrifice what may be a harmless cow rather than to risk infecting the others with a mastitis. In the case of two valuable animals a quarantine has been established and their milk examined daily for months, indeed during almost the entire period of lactation. One such developed an udder inflammation which seemed to be chronic, and the other, tuberculosis.

Among the conclusions reached by Harris in his study of this subject it is stated that, "Particularly, should more attention be given to the veterinary inspection of the cows' udders, with less absolute dependence upon laboratory examination of milk for signs of infectious processes." It is both necessary and desirable that the care of dairy animals be supervised by a veterinarian, and it has been the experience of this dairy that the constant laboratory examinations have stimulated a more careful and close observation of the animals by those having the practical care of them, both the veterinarian and the herdsman. Upon the herdsman, far more than upon the veterinarian, who could scarcely be expected to examine a large herd daily, must rest the responsibility of the detection of the first signs of disease. Frequently the finding of numerous cells or streptococci indicates to him a course of action which, had he waited for the usual clinical signs, would have been deferred to the detriment of the animal. For example, a cow having been in the herd for more than two years, and possessing a clean laboratory record for that length of time, suddenly gave a milk containing many cells. Examination showed what the milker had failed to see, namely an inflamed spot at the junction of one teat with the udder. This developed into a boil. Five days after the first examination pus and streptococci in abundance were in the milk from the sore teat and streptococci were in the milk from the other three quarters. When the pus was first noted in the labora-

tory the lesion, clinically, was so inconspicuous that only careful looking found it, and without the pus report, in all probability, it would not have been noticed for several days.

Of considerable interest in this connection is the occurrence of pus in the milk of cows several days before the lesions of cow pox make their appearance. Indeed, the association of cow pox with pus in milk, accompanied occasionally by streptococci, has been an exceedingly interesting phase of the study of this herd. The barns had been unusually free of pox until the autumn of 1905 when one or two cows were found with well-marked lesions. Then it spread rapidly and many cases developed.

A comparison of laboratory findings with those of the herdsmen showed, occasionally, pus reports from cows normally clean several days before pox appeared macroscopically. A typical case of this kind has the following history: A cow examined on November 28 and December 6 gave clean milk; on December 14 pus in quantity was found, and again on December 15 and 16 the same condition prevailed. On December 18 there were signs of pox, which were unmistakable on December 19. This was a severe case and very persistent leaving finally a scaly udder which was difficult to keep clean. The condition did not improve and the cow was sold in August of 1907.

A study of 16 cases of pox, both mild and severe, showed pus in nine of them. Generally this seemed to come from all the quarters, but two cases were found in which one quarter, only, was affected. Five of the 16 cases showed streptococci with the pus, and in one case, which was very severe, both streptococci in long chains and staphylococci in abundance were observed.

The external evidence is by no means an indicator of the internal condition. Some cows with marked lesions, if milked carefully and with proper precautions, give a milk free from cells; others with mild cases, no matter how carefully they are handled, may persist in giving a milk rich in cells.

A phase of the question of leucocytes in milk which would be of great value is the relationship, if there is any, between their presence there in increased numbers and an invasion of the animal by a tuberculous infection. When the udder itself is the seat of the lesion one

would naturally expect evidences of tissue disintegration in the milk, but this would not, of necessity, follow an invasion of other tissues.

We have observed 14 cows which have failed to pass the tuberculin test. The great majority of them, however, though purchased with a tuberculin test certificate, were condemned a few months after buying; hence the laboratory records are short. Of the 14, four never showed an increase of leucocytes; six had scattering leucocyte counts, but not sufficient to cause uneasiness; four had pronounced leucocytosis and had been carefully watched on this account for signs of disease, but none were noted. For three of these cows, two of which were under observation for a longer period than the others, the following brief histories are given:

No. 5. Fresh in October, 1906. Ten successive laboratory examinations, covering three months, showed a clean milk. Then she had pus occasionally, and in the fifth month there was an inflammation of one quarter with pus and a trace of blood in the milk. From this time on until July, when she was condemned for tuberculosis, she showed scattering pus counts, 19 examinations giving eight positive results. At autopsy the tubercular lesions were found in the intestines only.

No. 25. This animal gave pus in her milk every two or three weeks during the whole period of lactation, but there were never any clinical findings to account for its presence. She was dry for two months, then calved normally, but again showed pus frequently. Before the second period of lactation was ended she failed to pass the tuberculin test.

No. 80. Bought November 24, 1906. Blue grade. Fresh. Laboratory examinations began on November 30, when pus in quantity was reported. Between this date and July 6, 1907, her milk was examined twenty times. On December 7 a second pus report from the laboratory caused a careful examination of the cow's udder and it was found that there was an incipient case of pox. This was well developed on December 19, when again pus in quantity was found. A fortnight later the pox was cleaned up and her milk was almost free of leucocytes. Between this time and July 6 15 laboratory examinations were made seven of which showed pus in quantity, but very careful search, clinically, failed to show any abnormality. However, the cow failed to pass the July tuberculin test and was slaughtered. There were no lesions found in the udder.

The foregoing statement of the laboratory findings in so far as streptococci and increased leucocytes are concerned and their correlation, when possible, with the condition of the animal clinically, is of necessity incomplete. The work is in its infancy and the results which we have obtained may be interpreted in various ways. They are offered, however, with the hope that others studying the production of pure milk will give their co-workers the benefit of their observa-

tions, since only by broad researches, preferably on herds which are purely milk producing, can some of the questions now unanswered be settled.

Whether laboratory findings are or are not to be rigidly accepted by the herdsman we believe that they tend toward the production of a clean milk.

The milk of the herd which we have reported is examined daily, also, for the number of organisms. It is plated each day when about six hours old, having been sent to the laboratory directly from the farm, and the milk of the same milking is again plated when received from the dealer and is from 26 to 40 hours old. In this way trouble either at the farm or in the hands of the milk dealer in the city can be traced. Each month an average of all the counts made during the preceding four weeks is struck. Table 2 gives such averages for 13 months. These figures represent over 500 examinations.

TABLE 2.

Month	Milk from Dairy 6 hours Old	Milk from the dealer from 27 Hours to 40 Hours Old
1906-October.....	3,408	3,847
November.....	3,370	3,060
December.....	2,372	2,300
1907-January.....	2,768	2,412
February.....	4,127	4,881
March.....	2,426	2,227
April.....	1,576	1,694
May.....	2,818	3,997
June.....	3,554	3,133
July.....	1,774	2,565
August.....	2,166	4,400
September.....	4,218	6,481
October.....	2,974	1,373

## SUMMARY.

A correlation of the finding of streptococci or of many leucocytes in milk by the laboratory, with the physical condition of the cows in a herd maintained for the production of a very clean milk, would seem to show that in many cases there is a connection between such findings and the condition of the cow, both in relation to specific udder and to systemic affections. Such parallelism seems to obtain for the end of the lactation period, for the beginning of an udder inflammation, for an attack of cow pox, and, possibly, for chemotactic con-

ditions due to high feeding of animals constitutionally unable to transform the increased feed into increased milk.

The frequent laboratory examination of the milk of individual cows has materially assisted the herdsman in preserving the good health of the animals and has stimulated more frequent and careful clinical observations.

It is believed, too, that such examinations are a very material factor in maintaining a bacterial count which, for the past 13 months, averages 3,267 organisms per c.c. when the milk is from 26 to 40 hours old.

Our cordial thanks are due to Dr. E. Q. St. John, who has most ably assisted in the microscopic and bacteriological work needed for this paper.



## A REVIEW OF ANAPHYLAXIS, WITH ESPECIAL REFERENCE TO IMMUNITY.\*†

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ANAPHYLAXIS (*ἄνα*, against, and *φυλός*, guard, or *φυλάξις*, protection), also called hypersusceptibility, supersensitiveness, is a condition of unusual or exaggerated susceptibility of the organism to foreign substances. The word *anaphylaxis* suggests the contrary condition to *prophylaxis*; it may be congenital or acquired; it is specific in nature. The condition of anaphylaxis may be brought about by the introduction of any strange proteid into the body. Hypersusceptibility to proteins that are non-poisonous in themselves may readily be induced in certain animals.

An animal may be in a condition of hypersusceptibility and immunity at the same time. The two conditions are closely interwoven. The one may be dependent upon the other. von Pirquet advises that the term "immunity" be limited to indicate the condition of complete resistance in which no clinical reaction occurs, when poisons (such as diphtheria, tetanus, etc.) are introduced into the organism. He suggests the term "allergie" to indicate conditions of acquired immunity associated with anaphylaxis, such as that induced by vaccinia against variola, that of the luetic against syphilis, or that produced by one attack of some of the acute specific infections. This condition of allergic manifests itself in the renewal of the infection in an entirely different manner from the reaction to the primary infection.

The tuberculin and mallein reactions are well-known instances of anaphylaxis. These substances are not poisonous when introduced into a healthy individual, but the tuberculous individual is anaphylactic to tuberculin, and an individual suffering with glanders is in a state of hypersusceptibility to mallein.

The best-studied instance of anaphylaxis is that produced in the guinea-pig by the injection of a foreign proteid, for example, horse

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serum, egg white, milk, etc. Especial study has been made of the anaphylactic action of the blood serum of the horse, partly because that serum is so much used in serum therapy.

It has long been known that the blood of certain animals is poisonous when transfused or injected into certain other species. Many instances might be cited showing that the blood serum of an animal of one species has poisonous properties when injected into an animal of another species. But the blood serum of the horse apparently lacks such poisonous action. Very large quantities of the blood serum of the horse may be injected into man, rabbits, guinea-pigs, and many other animals without serious inconvenience, except occasionally a slight reaction at the site of inoculation.

In a certain proportion of cases the injection of horse serum into man is followed by urticarial eruptions, joint pains, fever, swelling of the lymph nodes, edema, and albuminuria. This reaction, which appears after an incubation period of 8 to 13 days, has been termed by von Pirquet and Schick the "serum disease."

In exceptional instances sudden death has followed an injection of horse serum in man.

We have shown that ordinarily horse serum is a comparatively bland and harmless substance when injected into certain animals; but these animals may be rendered so susceptible that an injection of horse serum may produce death or severe symptoms. For example, large quantities of horse serum may be injected subcutaneously into the peritoneal cavity, into the brain, or directly into the circulation of a guinea-pig without apparently causing the animal the least inconvenience. However, if a guinea-pig is injected with a small quantity, say  $1/250$  c.c., of horse serum and after the expiration of a certain interval is again injected with horse serum the result will probably be fatal. The first injection of horse serum has sensitized the animal in such a way as to render it very susceptible to a second injection of horse serum.

A certain time must elapse between the first and second injections before this toxic action is able to manifest itself. This "period of incubation" is from 10 to 12 days, and corresponds suggestively with the period of incubation of the serum disease which von Pirquet and Schick place at 8 to 13 days.

Guinea-pigs may be sensitized with exceedingly small quantities of horse serum. In most of our work we used quantities less than 1/250 c.c. and we found in one instance that 1/1,000,000 c.c. of horse serum was sufficient to render a guinea-pig susceptible.

It also requires very small quantities of horse serum, when given in a second injection, to produce poisonous symptoms. One-tenth c.c. injected into the peritoneal cavity is sufficiently to produce serious symptoms.

While at first we thought that diphtheria antitoxin had some relation to this action, we are now able to state positively that it has nothing whatever to do with the poisonous effects of horse serum; further, that diphtheria antitoxin in itself is absolutely harmless. The toxic action which we have studied is caused by a principle in normal horse serum and is entirely independent of the antitoxic properties of the serum.

*Historical.*—The fact that guinea-pigs which had been used for the testing of diphtheria antitoxin frequently died when later given an injection of serum has been known almost since the discovery of diphtheria antitoxin, but no one seems to have attached any significance to the relation between the two injections until the fall of 1905. Most of the workers with serum regarded it as an accident pure and simple or considered that the animal's vital resistance had been lowered by the first treatment; some even thought that it was the effect of cold, as the sera were usually kept in the ice chest and were injected at once after removing from the ice box. Theobald Smith, however, evidently had the problem in mind as he spoke to Ehrlich about it when the latter was in this country in 1904, the result being Otto's work. When we first encountered the phenomenon it at once occurred to us that there might be some relation between it and sudden death after the injection of antitoxin in man, and our work was begun in the hope of throwing light on this unfortunate accident. The results of our work on this most interesting subject have been published in a number of papers<sup>1</sup> since April, 1906. We foresaw early in our work that the phenomenon very probably had a close relation to immunity and our recent work with proteids obtained from the bacterial cells has shown this to be true. We have been able to produce anaphylaxis in guinea-pigs with bacterial extracts and have found that these animals possess an immunity to the corresponding infections.

Early in the last century Magendie<sup>2</sup> (1839) found that rabbits which had tolerated two intravenous injections of egg albumin without any ill effects immediately succumbed to a further injection made after a number of days. Later, workers with precipitin

<sup>1</sup> M. J. Rosenau and John F. Anderson, *Bull. No. 29*, Hyg. Lab., U. S. Pub. Health and Mar.-Hosp. Serv., Washington, 1906; *Jour. Med. Res.*, 1906, 10, p. 179; *Jour. Amer. Med. Assn.*, 1906, 47, p. 1007. See also John F. Anderson, *Bull. No. 30*, Hyg. Lab., U. S. Pub. Health and Mar.-Hosp. Serv., Washington, 1906; *Jour. Med. Res.*, 1906, 10, p. 241; *Ibid.*, p. 259.

M. J. Rosenau and John F. Anderson, *Bull. No. 36*, Hyg. Lab., U. S. Pub. Health and Mar.-Hosp. Serv., Washington, 1907; *Jour. Med. Res.*, 1907, 11, p. 381; *Jour. Infect. Dis.*, 1907, 4, p. 552.

<sup>2</sup> German translation by Krupp, Leipzig, 1839; cited by Ehrlich, *Col. Stud. Imm.*, p. 332.

have frequently found that some of their animals died suddenly during the course of treatment from no apparent cause, while what really happened was they were in a state of anaphylaxis to the foreign proteid. Other analogous instances may be found scattered throughout the early literature.

Knorr<sup>1</sup> (1895) found that guinea-pigs develop an increasing sensitiveness to tetanus toxin.

Hericourt and Richet<sup>2</sup> (1898) in studying the effects of eel serum on dogs found that they were not able to immunize them against the serum, but that on the contrary there was an increasing sensibility to it so that finally the dogs died.

Behring and Kitashima<sup>3</sup> (1901) repeating some of Knorr's work, also found an increasing sensitiveness to tetanus toxin on the part of guinea-pigs.

Portier and Richet<sup>4</sup> (1902) found that if dogs were given a very small dose of a glycerin extract from the tentacles of actinia, and then in 15 or 20 days given a second small dose, the animals quickly succumbed. The dose given was so small as to cause no symptoms in a normal animal. They appear to be the first to use the word "anaphylaxis" to indicate hypersensitiveness to a poison.

Arthus<sup>5</sup> (1903) studied the effect of repeated injections of horse serum on rabbits and found that if the rabbit be given repeated injections of horse serum at some days interval it caused, even in small doses, results which, according to the number of the previous injections and methods of injection, were local or general and benign or grave.

Richet<sup>6</sup> (1904-5) in studying two poisons (congestin and thalassin) extracted from actinia found that if a very small dose, which caused practically no symptoms in a dog, was followed after 22 days by another small dose the animal become very sick or quickly died.

Pirquet and Schick<sup>7</sup> in 1905, in a monograph upon the serum disease described in detail this syndrome which sometimes follows injections of horse serum in man. They show that the symptoms of this "disease" when caused by a second injection may either appear at once (the immediate reaction) or after a shortened period of incubation (the accelerated reaction). From these clinical observations Pirquet and Schick draw original and far-reaching conclusions. They show the relation of these clinical observations to the phenomenon of hypersusceptibility, and indicate the importance of these facts in general pathology. They draw attention to the analogy to the tuberculin reaction as a well-known instance of hypersusceptibility. Pirquet and Schick believe that the serum reactions give a possible explanation of the period of incubation of infectious diseases and finally conclude that the immunity caused by vaccinia and a group of infections is due to the power of immediate reaction acquired by the organism.

During Ehrlich's visit to this country in 1904, Theobald Smith told him that guinea-pigs which had been used in testing the potency of diphtheria antitoxin become acutely sick or die if injected subcutaneously several weeks later with several cubic centimeters of normal horse serum. Ehrlich gave the problem to Otto, who worked out many

<sup>1</sup> *Habilitationschrift*, 31 pp., 8 pls., 8 vo; Marbourg, 1895.

<sup>2</sup> *Compt. rend. soc. biol.*, 1898, 53, p. 137.

<sup>3</sup> *Berl. klin. Wchenschr.*, 1901, 38, p. 157.

<sup>4</sup> *Compt. rend. soc. biol.*, 1902, 54, p. 170.

<sup>5</sup> *Compt. rend. soc. biol.*, 1903, 55, p. 817.

<sup>6</sup> *Arch. di fisiol.*, 1904, 1, p. 129. *Compt. rend. soc. biol.*, 1905, 58, p. 109. *Ibid.*, p. 112.

<sup>7</sup> Dr. C. von Pirquet and Dr. B. Schick, *Die Serumkrankheit*, Leipzig, 1905.

of the essential features of the phenomenon. This article,<sup>1</sup> published late in 1905, came to our attention while we were correcting the page proofs of our first paper upon the same subject. It seems that the work was done about the same time, but Otto's paper appeared first. Otto showed what is now well known to be the result of a second injection of horse serum into guinea-pigs. He demonstrated that the diphtheria poisons play no part in the phenomenon. However he found that guinea-pigs first treated with mixtures of diphtheria toxin and serum are more susceptible than those treated with serum alone. Otto showed further that immunity to the poisonous action of the serum injection may be acquired by repeated injections of large amounts of serum at short intervals. He demonstrated that this hypersusceptibility bears no relation to the specific precipitins. Finally Otto discusses the relation of the Theobald Smith phenomenon to the cases of reinjection in man and cites instances of alarming symptoms following the second injection of antitoxic horse serum.

Many phases of the subject were studied by Rosenau and Anderson<sup>2</sup> late in 1905 and early in 1906. They studied particularly serum anaphylaxis in the guinea-pig. They showed that a single injection of horse serum was harmless to a normal guinea-pig but that a second injection after a definite interval was usually fatal. This "period of incubation" was found to be about 10 days. The poisonous property was shown to have absolutely no connection with the *antitoxic* property of the serum or with the diphtheria *toxins*.

The poisonous principle in serum was found to be quantitatively specific, death only occurring at the second injection with the homologous serum and never with the heterologous. Hemolysis and precipitin formation were excluded as factors. The poisonous property was unaffected by various chemical, physical, and mechanical influences and by age. Guinea-pigs may be sensitized with 1/1,000,000 c.c. of serum and they remain sensitive for a very long time. They were able to sensitize guinea-pigs by feeding them horse meat or horse serum. They showed further that the offspring of sensitive female guinea-pigs were sensitive to a *first* injection of serum; this being the first instance of the experimental demonstration of the hereditary transmission of a hypersusceptibility or tendency to a disease.

Anderson<sup>3</sup> found that female guinea-pigs could transmit to the same offspring hypersusceptibility to horse serum and immunity to diphtheria toxin. This fact is of great importance in testing antitoxic sera and necessitates care in the selection of breeders for guinea-pigs to be used in serum work.

McClintock and King<sup>4</sup> (1906) gave 10 guinea-pigs from 1/250 to 1 c.c. of horse serum by the stomach and 13 days later 6 c.c. of serum, either subcutaneously or intraperitoneally, without causing symptoms in any of them. They conclude that the sensitizing action of horse serum given by the mouth is not nearly so great as when given subcutaneously or intraperitoneally.

Currie<sup>5</sup> (1907) has studied the effect of repeated injections of horse serum in persons admitted for treatment in the city of Glasgow Fever and Smallpox Hospital at Belvidere. He concludes that it is apparent from the facts detailed by him that

<sup>1</sup> R. Otto, "Das Theobald Smithsche Phänomen der Serumüberempfindlichkeit," *v. Leuthold-Gedenkschrift*, I Band, 1905.

<sup>2</sup> *Bull. No. 29*, Hyg. Lab., U. S. Pub. Health and Mar.-Hosp. Serv., Wash., 1906.

<sup>3</sup> *Bull. 30*, Hyg. Lab. U. S. Pub. Health and Mar.-Hosp. Serv., Wash., D. C., 1906.

<sup>4</sup> *Jour. Infect. Dis.*, 1906, 3, p. 700.

<sup>5</sup> *Jour. Hyg.*, 1907, 7, p. 35.

repeated injections of horse serum induce symptoms of supersensitization in man, but it is also apparent that the same facts lend no countenance to the suggestion that the death of persons suffering from diphtheria is to be apprehended as the result of repeated injections of antidiphtheric serum.

Besredka and Steinhardt<sup>1</sup> (1907) studied with much care certain features of hypersusceptibility to horse serum in guinea-pigs; they note that the French sera are much less toxic than those used by Otto in Frankfurt and the sera used by us. Besredka and Steinhardt had a mortality of about 25 per cent when 5 c.c. of serum was given intraperitoneally at the second injection, whereas death was the rule in our experiments under similar conditions. Most of their work was done with doses of 0.05 to 0.25 c.c. given directly into the brain, which either killed or caused grave symptoms in susceptible guinea-pigs. Besredka and Steinhardt lay stress upon the production of "antianaphylaxis," which we termed "immunity." They found that a single injection of serum given into the peritoneum of a sensitized guinea-pig conferred immunity to a subsequent injection of 0.25 c.c. into the brain; in one case the antianaphylaxis was present one and a half hours after the injection into the abdominal cavity. They were unable to demonstrate any protective properties in various organs of immune guinea-pigs.

Nicollé<sup>2</sup> (1907) found that guinea pigs were not susceptible to the necrotic action induced by repeated injections of horse serum, as is the case in rabbits. He also found that daily injections or "spaced" injections, after the method of Arthus, did not induce a high degree of hypersusceptibility in guinea-pigs.

Besredka<sup>3</sup> (1907) questions whether we should not consider this toxic property of horse serum, in human therapy. He suggests that an antitoxic serum, 0.05 c.c. of which when given into the brain will kill or cause grave symptoms in a sensitive guinea-pig, should be considered as above the average toxicity and ought to be excluded from use in man.

Rosenau and Anderson<sup>4</sup> (1907) in a further contribution to the subject studied particularly the relation of anaphylaxis to immunity. In addition to confirming and extending their work on the specific nature of the phenomenon they made further observations on the relation of various physical influences and chemical substances on the reaction. Among other things they brought out the fact that proteids extracted from the bacterial cells and injected into guinea-pigs produced on the second injection the same train of symptoms as in the case of serum anaphylaxis. It was found that in certain instances the hypersusceptibility produced by these bacterial extracts left the animal immune to the corresponding infection.

Vaughan<sup>5</sup> (1906) advanced the theory that the first injection of the strange proteid is broken up into components, one of which is toxic, but that the animal is not poisoned because this breaking up takes place slowly. The cells, however, learn from this lesson how to break up the complex molecule, so that when more of the strange proteid is introduced at the second injection it is violently rent asunder, quickly liberating large quantities of the toxic principle of the complex molecule.

Vaughan and Wheeler<sup>6</sup> (1907) have elaborated this explanation by further studies

<sup>1</sup> *Ann. de l'Inst. Past.*, 1907, 21, p. 117.

<sup>2</sup> *Ann. de l'Inst. Past.*, 1907, 21, p. 128.

<sup>3</sup> *Compt. rend. soc. biol.*, 1907, 62, p. 477.

<sup>4</sup> *Bull. No. 36, Hyg. Lab.*, U. S. Pub. Health and Mar.-Hosp. Serv., Wash., 1907.

<sup>5</sup> V. C. Vaughan, *Jour. Amer. Med. Assn.*, 1906, 47, p. 1000.

<sup>6</sup> *Jour. Infect. Dis.*, 1907, 4, p. 470.

upon egg-white and bacterial proteids split into poisonous and non-poisonous portions. These authors believe that when egg-white, or the non-poisonous portion of egg-white, is injected into a fresh animal certain cells of the body are so influenced that they elaborate a new ferment, which, in the form of zymogen, remains in the cell until activated by the second injection, when it is set free and splits up the egg-white in a manner similar to that used by Vaughan in the laboratory. Vaughan and Wheeler believe that the effect induced in the animal is the same as that caused by the poisonous portions of egg-white as they have split it up in the retort.

Gay and Southard<sup>1</sup> (1907) found in guinea-pigs dying from a second injection of serum and in those which had severe symptoms and were later chloroformed, what they considered characteristic lesions. Considerable hemorrhages, rather definitely localized, are the characteristic gross lesion. The hemorrhages may be in one or several organs, gastric hemorrhages being especially frequent. Microscopically there are in addition to the naked eye hemorrhages minute interstitial and oozing hemorrhages. They also claimed to have found fatty changes in voluntary muscle fiber, heart muscle fibers, and in nerve fibers.

Their explanation of serum anaphylaxis in the guinea-pig is as follows: There is a substance in horse serum (anaphylactin) which is not absorbed by the guinea-pig tissue, is not neutralized, and is eliminated with great slowness from the body. When a guinea-pig is injected with a small amount of horse serum the greater part of its elements are quickly got rid of; the anaphylactin remains and acts as a constant irritant to the body cells, so that their activity for the other elements of horse serum is greatly increased. At the end of two weeks of constant stimulation by the anaphylactin a condition is arrived at where if the cells are suddenly presented with a large amount of horse serum they are overwhelmed in the exercise of their increased assimilating functions, and functional equilibrium is so disturbed that local or general death may occur.

Besredka and Steinhardt<sup>2</sup> (1907) found that guinea-pigs could be put in a state of antianaphylaxis by the injection of horse serum into the brain as well as into the peritoneal cavity. They consider it a phenomenon of the same order as the disintoxication *in vitro* of the tetanized brain by antitetanic serum. They found that guinea-pigs could not be sensitized by intracerebral injection.

They think that their results seem to indicate that the phenomena of anaphylaxis and antianaphylaxis are similar to the precipitating and absorbing actions which govern the relation of colloids among themselves.

Besredka<sup>3</sup> (1907) confirms the work of Rosenau and Anderson that the toxicity of horse serum is destroyed by heating to 100° C.; he further states that it is markedly attenuated by varying degrees of temperature down to 55° or 50° C. He found that injection of calcium chloride the day before prevented the appearance of anaphylactic symptoms.

In a second paper, Otto,<sup>4</sup> in 1907, showed that animals may be sensitized by injecting them with the serum of sensitized animals. He believes that the first injection results in a weakening or disappearance of the portions ("rests") of the antigens which are in the body, and thus an apparent "hypersusceptibility" results. The duration of this hypersusceptibility depends upon the amount of serum injected the first time.

<sup>1</sup> *Jour. Med. Res.*, May, 1907, 16, p. 143.

<sup>2</sup> *Ann. de l'Inst. Past.*, 1907, 21, p. 384.

<sup>3</sup> *Compt. rend. soc. biol.*, 1907, 62, p. 1053.

<sup>4</sup> *Munch. med. Wchnschr.*, 1907, 55, p. 1665.

Richet<sup>1</sup> (1907) gives a general review of the subject of anaphylaxis and also some very interesting work on anaphylaxis produced by a substance obtained from the *mytilus edulis*. He found that the blood of a dog sensitized by this substance when injected into an untreated dog sensitized the animal two days later to an injection of the extract.

He thinks that anaphylaxis is due to the presence of a toxicogenic substance, non-toxic of itself, but producing a poison by reaction with the second injection of the extract. In support of this view he states that *in vitro* a mixture of the serum of a sensitized dog and of the extract is more toxic than the extract alone.

Goodall<sup>2</sup> (1907) gives observations on 90 patients who had received two injections of horse serum; of these 43.4 per cent gave either an immediate or accelerated reaction.

Besredka<sup>3</sup> (1907) concludes that the toxicity of therapeutic sera may be measured by means of intracerebral injections into sensitized guinea-pigs.

Measured in this way, different sera show a wide gamut of toxicity, the fatal dose varying from 1/4 to 1/128 c.c. This toxicity resides in the serum and not in the cellular elements.

The sera of horses living under apparently the same conditions have about the same toxicity; individual variations are rare and of little importance.

The difference in the toxicity of sera appears to be due, in the first place, to their origin; and in the second place, to their age.

Sera are hypertoxic on the day of bleeding, and gradually lose their toxicity. This loss, rapid at first, becomes gradual after the tenth day.

All therapeutic sera should be considered toxic within two months of bleeding.

In a general way, all sera that excite grave anaphylactic phenomena in doses of 1/16 to 1/20 c.c., and *a fortiori*, above this amount, should be considered toxic.

Besredka finally states that the technic and dosage by the intracerebral method is rapid, simple, and not expensive.

#### SYMPTOMS CAUSED BY THE INJECTION OF HORSE SERUM INTO A SENSITIZED GUINEA-PIG.

Very characteristic symptoms are produced by horse serum, either normal or antitoxic, when injected into a susceptible guinea-pig. The symptoms are apparently the same whether the injection is made subcutaneously or into the peritoneum, or whether normal or antitoxic horse serum is used. In five or ten minutes after injection the pig manifests indications of respiratory embarrassment by scratching at the mouth, coughing, and sometimes by spasmodic, rapid, or irregular breathing; the pig becomes restless and agitated. This stage of exhalation is soon followed by one of paresis or complete paralysis. The pig is unable to stand or, if it attempts to move, falls upon its side; when taken up it is limp. Spasmodic, jerky, and convulsive movements now supervene.

<sup>1</sup> *Ann. de l'Inst. Past.*, 1907, 21, p. 497.

<sup>2</sup> *Jour. Hyg.*, 1907, 7, p. 607.

<sup>3</sup> *Ann. de l'Inst. Past.*, 1907, 21, p. 777.



Pigs in this stage with complete paralysis may fully recover, but usually convulsions appear, and are almost invariably a forerunner of death. Symptoms appear about 10 minutes after the injection has been given; occasionally in pigs not very susceptible they are delayed 30 to 45 minutes. Only in one or two instances of the several hundred pigs which we have observed have the symptoms developed after one hour. Pigs developing symptoms as late as this are not very susceptible and do not die. The chain of symptoms is exceedingly characteristic. The symptoms do not always follow in the order given. Death usually occurs within an hour and frequently in less than 30 minutes.

If the second injection be made directly into the brain, the symptoms are manifested with explosive violence, the animal frequently dying within two or three minutes. The same is also true if the second injection be made directly into the circulation.

#### SENSITIZING PRINCIPLE.

We have advanced the theory that the substance which sensitizes the guinea-pig is perhaps the same as that which later poisons it. Profound chemical changes, perhaps in the central nerve cells, are probably produced by the first injection.

We devoted much time to the isolation of this sensitizing principle, without success.

It is unaffected by the various preservatives used for the preservation of antitoxic serum, by drying, by precipitation with ammonium sulphate or magnesium sulphate, or by admixture with diphtheria or tetanus toxins. Formaldehyd likewise has no influence upon this principle. Serum heated to 60° C. for 30 minutes is as potent in sensitizing animals to a subsequent injection as the unheated serum.

The removal of the spleen or the thyroid from the animal before or after receiving the sensitizing dose apparently has no influence upon the development of anaphylaxis.

Gay and Southard first pointed out that if the blood of a guinea-pig which has received a small sensitizing dose of normal horse serum be drawn, the serum collected, and 1.5 c.c. of this be given to a normal guinea-pig, the normal animal is rendered susceptible to a subsequent injection of horse serum 15 days later. This shows that the sensi-

tizing substance, or *anaphylactin* as it is called, is present in the blood serum of a sensitive animal. It must be present in an exceedingly minute amount, for we have shown that the blood of guinea-pigs receiving only 1/500 c.c. of serum contains this substance several months later, and 1.5 c.c. of the serum of such an animal, when injected into a normal animal, renders it sensitive to a subsequent injection 24 hours later of horse serum.

Guinea-pigs may be sensitized by either large or small amounts of serum. Amounts as great as 10 c.c. have sensitized the animals, while amounts as minute as 1/1,000,000 c.c. did likewise. The optimum sensitizing amount, however appears to be from 1/100 to 1/1,000 c.c.

It has appeared to us in our work that the animals are sometimes rendered slightly more sensitive if given a mixture of toxin-antitoxin used in testing serum. This may be due to the fact that the toxin, which is not completely neutralized by the serum, or which becomes disassociated, may lower the resistance of the animal to some extent. Guinea-pigs are sensitized if the injection be given subcutaneously, intraperitoneally, directly into the heart, into the brain, or if fed by the mouth.

#### THE TOXIC PRINCIPLE.

At one time we made efforts to isolate the active principle in horse serum which causes the symptoms, but as soon as we realized that the toxic principle present in horse serum exerts its action in quantities so minute as to place it almost in the category of the ferments, we realized how hopeless it would be with present technic to isolate this substance. Nevertheless, we devoted much time and study to the relation of this toxic principle to various chemical, physical, and electrical influences. The practical importance of eliminating this toxic principle from horse serum, or of neutralizing it, is at once evident.

We heated serum for various lengths of time, and found that the toxic property is entirely destroyed by heating to 100° C. for 25 minutes.

The toxic principle is not affected by various chemicals such as calcium chlorid, sodium nitrate, magnesium sulphate, ammonium sulphate, and formaldehyd. It is not affected by various ferments, alkaloids, and similar substances, such as taka diastase, pancreatin,

rennin, myrosin, invertin, emulsin, pepsin in acid solution and in alkaline solution, ingluvin, malt, papain, stropin, strychnin, morphin, and caffen.

It is not affected by freezing at 15° F., nor by filtration through porcelain, drying, precipitation and dialysis, and exposure to the X-rays.

We found it interesting to compare the toxic effects upon sensitive animals of untreated antitoxic serum, and the precipitated refined antitoxin; bulk for bulk we found them equally toxic. But as the same number of units can be given in half the bulk there is a manifest advantage in using the precipitated serum, as the rashes and other untoward effects of serum depend to some extent upon the volume of serum administered.

The smallest amount of serum given intraperitoneally that we have found to cause death of a guinea-pig is 0.1 c.c. One hundredth of a c.c., when given directly into the heart, is sufficient to cause the death of the animal, while 0.25 c.c. given into the brain is almost invariably fatal. In most of our work, however, we have used five or six c.c. of serum intraperitoneally, and this seems to be the favorite dose of other workers. Certain symptoms in guinea-pigs caused by a second injection of the serum suggested to us that the action might be due to hemolysis or the formation of precipitins. By a large number of experiments, however, we were able to exclude both hemolysis and the formation of precipitins as factors

#### THE INFLUENCE OF TIME.

It is necessary that a certain time elapse between the first and second injections of the foreign proteid before the toxic action is manifest. This "period of incubation" is from 10 to 12 days and corresponds suggestively with the period of incubation of the serum disease which von Pirquet and Schick place at 8 to 13 days and with the period of incubation of some of the infectious diseases.

If a guinea-pig be given an injection of serum and then be injected again any time before the eighth day no ill results follow. In other words, the animal has not had time to enter a state of anaphylaxis to the foreign proteid. If, however, the injection be given after the eighth or tenth day the animal is then in a state of anaphylaxis or

hypersusceptibility to the foreign proteid. The shortest interval between the two injections in which we have found death to occur is 12 days. The animal, however, remains susceptible a very long time. The longest period which we have observed is 732 days between the first and the second treatment. We have no doubt, however, that the animal remains susceptible during its entire life.

#### THE SPECIFIC NATURE OF ANAPHYLAXIS.

From our first studies upon hypersusceptibility we were interested in the question, "Is this reaction specific?"

In our work upon the toxic action of horse serum, we showed that this reaction is quantitatively specific for serums. That is, guinea-pigs sensitized with horse serum are subsequently very susceptible to a second injection of horse serum, but only slightly if at all susceptible to a second injection of the serum of other animals, such as rabbit, cat, dog, hog, sheep, chicken, or man. Conversely, guinea-pigs sensitized with the serum of these other animals are not very sensitive to a second injection of horse serum, whereas, they respond actively to a subsequent injection of the same kind of serum as that used for the first injection.

We have further shown that the specific nature of this phenomenon is more marked when proteid substances of widely different nature are used at the first and second injections. Thus a guinea-pig sensitized with horse serum does not react to a subsequent injection of egg-white, vegetable proteid, or milk. A guinea-pig sensitized with milk does not react to the other proteid substances mentioned.

We have recently succeeded in demonstrating more clearly the specific character of the phenomenon we are studying by proving that guinea-pigs may be in a condition of anaphylaxis to three proteid substances at the same time. For instance, a guinea-pig may be sensitized with egg-white, milk, and horse serum, and subsequently react to a second injection of each one of these substances. The guinea-pig may be sensitized by injecting these strange proteids either at the same time or at different times, in the same place or in different places, or by injecting them separately or mixed. The guinea-pig differentiates each anaphylactic-producing proteid in a perfectly dis-

tinct and separate manner. The animal is susceptible to the second injection of each one of the three substances in the same sense that it is susceptible to three separate infectious diseases.

One of our animals reacted sharply to the second injection of milk. This guinea-pig presented the usual symptoms from which it gradually recovered. Five hours after the injection of milk, it was given an injection of egg-white, from which it died. These two reactions in so brief a time seem to accentuate the specific nature of the phenomenon we are studying. It also adds weight to our belief that profound chemical changes probably in the central nervous system, rather than morphologic alterations, explain the essential features of the reaction.

#### FEEDING EXPERIMENTS.

Guinea-pigs may be sensitized by feeding them meat or serum.

Uhlenhuth<sup>1</sup> found that when rabbits were fed with egg albumen by means of a stomach tube their blood, after a while, developed the power to precipitate egg albumen.

Metchnikoff<sup>2</sup> reports that he obtained immunization by feeding one animal with the blood of another species. He fed horses' blood to white rats and noted that after several weeks the serum of the rats developed decided agglutinative and hemolytic properties.

We found that guinea-pigs could be sensitized by feeding them for some days on horse meat, or dried horse serum, mixed with their food. We did not use a stomach tube, as the possibilities of making slight wounds in the esophageal or gastric mucosa would vitiate the feeding experiments, and we knew from our previous work that very small quantities could readily sensitize the animal to a subsequent injection of serum.

The guinea-pigs that had been fed with horse meat or horse serum, after an interval of at least 14 days, were injected with horse serum and reacted in a characteristic manner.

We also found that guinea-pigs could be sensitized to cattle serum by feeding them with beef. Cooking the meat entirely destroyed its sensitizing properties.

The fact that guinea-pigs may be rendered susceptible by the feeding of strange proteid matter opens an interesting question as to

<sup>1</sup> *Deut. med. Wchnschr.*, 1900, 26, p. 734

<sup>2</sup> *Centralbl. f. Bakt.*, 1901, 29, p. 531.

whether sensitive guinea-pigs may also be poisoned by feeding with the same serum given after a proper interval of time. If man can be sensitized in a similar way by the eating of certain proteid substances, may not this throw light upon those interesting and obscure cases in which the eating of fish, sea food, and other articles of diet habitually cause sudden and sometimes serious symptoms?

#### HEREDITARY TRANSMISSION.

In the course of our work we had the opportunity to test the susceptibility of the young of susceptible female guinea-pigs and we found that hypersusceptibility to the toxic action of horse serum is transmitted from the mother guinea-pig to her young. This function is solely maternal; the male takes no part whatever in the transmission of these acquired properties. Whether this maternal transmission is hereditary or congenital cannot be definitely stated.

We were able to exclude the milk as a factor in transmitting the hypersusceptibility to the toxic action of horse serum by a series of "exchange" experiments. Exchange experiments consist in at once placing guinea-pigs born of a susceptible mother to nurse with an untreated female, and in exchange, the young of the untreated female are at the same time placed to nurse with the susceptible female. From these exchange experiments we learn that the hypersusceptibility is not transmitted to the young in the milk.

We also learned from our experiments that hypersusceptibility may be transmitted from mother to young, whether the mother is sensitized before or after conception.

If an anaphylactic tendency is transmitted from mother to young in man it may explain the severe reaction and death that occasionally takes place following the first injection of serum.

These results upon the hereditary transmission of the susceptibility to the poisonous action of horse serum in guinea-pigs may throw light upon the well-known inherited tendency to tuberculosis in children born of a tuberculous parent.

There are certain analogies between the action of tuberculosis and horse serum. Both produce a hypersensitiveness and also a certain degree of immunity. Now that we have proved that this hypersensitiveness or anaphylactic action in the case of horse serum may be

transmitted hereditarily in guinea-pigs, may it not throw light upon the fact that tuberculosis "runs in families"? While there are several recorded instances demonstrating that immunity to certain infectious diseases may be transmitted from a mother to her young, this is, as far as we know, the first recorded instance in which hypersensitiveness, or a tendency to a disease, has been experimentally shown to be hereditarily transmitted from a mother to her young.

#### ACTION OF HORSE SERUM UPON MAN AND OTHER ANIMALS.

It may be that man cannot be sensitized in the same way that we have shown to be the case with guinea-pigs. We made no human experiments, but experimental data are recorded by others which have a direct bearing on this question.

Repeated injections of horse serum into man is not an infrequent occurrence. Patients suffering with diphtheria are often given injections of antitoxic serum at short and frequent intervals. It is also not rare for persons to have several attacks of diphtheria at long intervals and to be treated each time with antidiphtheric serum.

Certain sera, for example, the antitubercle serum of Maragliano, or the antirheumatic serum of Menzer, are habitually used by injections at intervals of days or weeks.

In all of these cases of frequent and repeated injections the amount which has been injected and the interval between the injections must be taken into account in relation to this work. Pirquet and Schick in their work on *Serumkrankheit* give eight instances in which children received two injections of horse serum at intervals of from 16 to 42 days between the first and second injections. All these eight cases show this in common, that after the first injection of horse serum, the symptoms of the serum disease appear after the normal period of incubation, namely, between the eighth to thirteenth day. But when the same individuals are again injected with horse serum after intervals of 16 to 42 days, there reappears at once, or at least within 24 hours, symptoms of the serum disease.

Von Pirquet and Schick further give a list of 60 children who were injected with antitoxic horse serum at intervals from six days to seven and a half years between the first and second injections. They found that when the second injection was given from 14 days to four months

after the first injection, they obtained, with great regularity, what they termed "the immediate reaction," but when the interval between the first and second injection is over four months they obtained little or no immediate reaction, but what they termed "an accelerated reaction," for the fever, urticaria, and other symptoms of the serum disease appeared on the fifth, sixth, seventh, or eighth day. It will be remembered that the normal period of incubation for the symptoms of the serum disease to appear after the first injection is between the eighth and thirteenth day. Von Pirquet and Schick lay special stress upon the phenomena of the "immediate" and "accelerated" reactions following the second injection.

We might also conclude, despite the suggestion in our work upon sensitizing guinea-pigs by feeding them with horse serum or horse meat, that children are not sensitized to the toxic action in horse serum by eating horse meat from the fact that horse meat is a favorite article of diet in certain European countries, and there is nothing on record to show that the infection of horse serum in those countries is fraught with more danger than where this practice does not obtain. We must, however, remember that our work has shown that guinea-pigs are sensitized with exceedingly minute quantities of the strange proteid, and that repeated injections cause an immunity; and it is possible that the same action may be true of feeding. Further, we have shown that cooking destroys the sensitizing property of meat.

Man reacts to the first injection of horse serum after a period of incubation of 8 to 13 days. Guinea-pigs show practically no reaction following the first injection. Both react to a second injection. The reactions in man and the guinea-pig, however, differ both in severity and kind. The relation, therefore, that our observations upon the guinea-pig may have in its application to man must await further study. Of course, the fact that other animals besides man and guinea-pigs react to a second injection of horse serum would seem to indicate that we are dealing with one and the same action.

We have tested monkeys, rabbits, mice, dogs, cats, rats, chickens, and pigeons to determine whether any of these animals may be sensitized to the action of horse serum. Thus far we have obtained a response in the dogs, rabbits, and cats.

Von Pirquet and Schick also found that the first injection into rab-



bits caused no clinical effect, but that subsequent subcutaneous injections caused immediate reaction in the production of local edema which extended even to gangrene. Second injections, when introduced intravenously, produced symptoms of collapse and even death.

Arthus also found that the injection of horse serum into rabbits caused no symptoms, whether the horse serum was injected subcutaneously or intravenously, but when he injected the serum every six days subcutaneously, he obtained, after the fourth injection, edema and local reaction which continued to gangrene.

A rabbit which von Pirquet and Schick had previously treated with eight injections was then given 2 c.c. of horse serum into the ear vein. The rabbit reacted to this in one minute and, after characteristic symptoms similar to those we see in guinea-pigs, died in about four minutes.

Arthus obtained similar results with repeated injections of sterilized milk.

#### IMMUNITY TO ANAPHYLAXIS.

We showed in our first publication on the subject of anaphylaxis that guinea-pigs may be *actively* immunized against this phenomenon. At the same time we demonstrated that the immunity could not be transferred *passively* to other animals in the blood serum or body juices.

Guinea-pigs may be actively immunized in several ways: (1) by repeated injections of serum during the period of incubation: that is, during the first 10 days, before the animal reaches the state of hypersusceptibility; (2) by recovery from a second injection given during the anaphylactic stage.

The fact that guinea-pigs cannot be immunized passively by the transference of blood or body juices would make it appear that the "immune body," if such exists against the toxic action of horse serum, is not free in the blood or body juices, as is the case in diphtheria. In fact it has been questioned whether the active immunity which we have described is an instance of true immunity, or a "refractory" condition, or even an actual return to the normal.

For instance, Besredka and Steinhardt speak of immunity to anaphylaxis as "antianaphylaxis." They state that this immunity is not analogous to that conferred by the specific sera against certain

bacteria and their toxins; also that the intraperitoneal injection of a serum is able to protect a sensitized guinea-pig against a cerebral injection given a few hours later is never seen in the case of the specific sera. Besredka and Steinhardt state that the immunity conferred by the specific bacterial antisera is very transient; it lasts generally a few weeks at the most. On the other hand they find that in the case of antianaphylaxis to the serum phenomenon, the time is much longer, viz., many months. They ask the question, Is not this immunity to anaphylaxis simply due to the return of the sensitized guinea-pig to a normal state—in other words, a process of desensitization?

Gay and Southard also doubt immunity to anaphylaxis and consider such guinea-pigs to be rather in a "refractory" condition due to a prolongation of the period of incubation in the case of a large initial dose, or of multiple doses, in proportion to the amount of serum injected.

Subsequent researches have strengthened our belief that we are dealing with a true condition of immunity and not a prolongation of the period of incubation or a return to the normal. Thus it has been shown that guinea-pigs in the "refractory" condition still contain anaphylactin in their blood. It is at once evident that they have not returned to their normal condition. Further, we have demonstrated that such "refractory" female guinea-pigs will transmit this anaphylactic substance to their young. Only the sensitizing substance passes into the blood of the foetus, which is therefore in a condition of hypersusceptibility. The "immunizing substance" or "condition" is not hereditary or congenital.

It seems to us that we have here a striking analogy to that phase or kind of immunity which von Pirquet describes as "allergie." In other words, we have an acquired immunity associated with anaphylaxis. In guinea-pigs this immunity may follow one attack of the disease, i. e., the serum reaction. As stated by von Pirquet, *allergie* manifests itself by an immediate reaction and corresponds to the condition of immunity conferred by an attack of smallpox or of some of the other acute infectious processes.

In the case of syphilis we have a striking instance in which the virus is not auto-inoculable. In the serum reaction in the guinea-pig an analogous train of events occurs, for after the sensitized guinea-

pig has responded, the reaction renders the organism immune. A somewhat similar immunity to hypersusceptibility is shown in the tuberculin reaction. Thus Vallée<sup>1</sup> has recently shown in studying the cutaneous reaction of tuberculous cattle, goats, and guinea-pigs that if tuberculous cattle be given a subcutaneous injection of tuberculin a few days before the cutaneous test is applied the latter reaction is prevented. Further, Calmette, Breton, and Petet<sup>2</sup> have found that rabbits free from tuberculosis, if given a small dose of tuberculin in the ear vein and then tested 16 hours later for the ophthalmic reaction to tuberculin, show a typical reaction; if the same rabbits are tested 48 hours later in the other eye the result is negative or very feeble, thus showing that an immunity to the reaction is acquired by the first reaction. This seems to be another example of allergy, or immunity to a specific hypersusceptibility.

#### HYPERSENSITIBILITY AND IMMUNITY PRODUCED BY BACTERIAL PROTEIDS.

We believe that the problem of hypersusceptibility has an important bearing upon the question of immunity, and hence we expressed the opinion that "resistance to disease may largely be gained through a process of hypersusceptibility. Whether this increased susceptibility is an essential element or only one stage in the process of resistance to disease, must now engage our attention." We cannot escape the conviction that this phenomenon of hypersusceptibility has an important bearing on the prevention and cure of certain infectious processes. Our work upon the hypersusceptibility produced by the bacterial proteids strengthens this belief, for our recent results prove that the phenomenon of hypersusceptibility to certain proteid substances extracted from the bacterial cell is followed by a definite immunity against infection by the micro-organism.

Experimental studies with the bacterial proteids are of the greatest importance on account of the practical uses to which results along this line may lead.

Hypersusceptibility may easily be induced in guinea-pigs with proteid extracts obtained from the bacterial cell. The first injection of most of the extracts used by us seems comparatively harmless to

<sup>1</sup> *Med. Vet.*, 84, p. 308.

<sup>2</sup> *Compt. rend. de soc. biol.*, 1907, 63, p. 296

the animal. A second injection of the same extract shows, however, that profound physiological changes have taken place. A definite period must elapse between the first and the second injection. The symptoms presented by the guinea-pigs as a result of the second injection resemble those caused by horse serum.

The phenomenon induced by a second injection is followed (in certain cases) by an immunity to the corresponding infection. The following is a summary of our experimental results along these lines.

*Extract of colon bacillus.*—The extract from the colon bacillus used in our experiments was obtained as follows:

A two-day-old culture of *B. coli communis* in Dunham's solution was used to heavily inoculate the surface of 84 large agar plates. These plates were grown at 37° C. for four days and the surface growth collected.

The bacterial mass was frozen 48 hours at about 15° F., thawed at room temperature, and then ground with sand by hand in a mortar for five hours, shaken vigorously half an hour, and again frozen 18 hours. After again thawing, the fluid was diluted with salt solution and filtered through a Berkefeld filter. The clear filtrate gave a distinct coagulum with heat and acetic acid.

All the other extracts were obtained by a similar process. In the case of the tubercle bacillus, the bacterial mass was first washed three days in running water to eliminate the soluble tuberculin as much as possible.

The hypersusceptibility induced by the colon extracts manifested itself by symptoms resembling those already described in the case of horse serum. The guinea-pigs scratched at the mouth with their hind legs. Most of them showed evidences of respiratory embarrassment by quickened, labored, or irregular breathing. Many of the pigs lay over on their sides, a common symptom. A few developed jerky movements; but in no case was convulsion noted. The pigs looked quite sick and ill at ease, but gradually recovered, so that by next morning they seemed normal.

Ten days following the second injection of the extract the guinea-pigs were given 5 c.c. of a heavy emulsion of colon bacillus from 24-hour-old agar slants, but showed no symptoms, and remained in good condition. Three controls received the same injection and died in twelve hours.

*Anthrax.*—A number of the guinea-pigs were given the extract from anthrax bacilli before infection; some were given a single injection, some two injections, and others daily injections for 20 days. Other guinea-pigs were given the extract used as a vaccine, both in single and repeated injections, after being infected with anthrax Bacilli. The extract did not seem to have any influence on the course of the disease, whether given before or after the infection.

*Tuberculosis.*—The guinea-pigs which have reacted to two injections of proteid extract obtained from the tubercle bacillus are now being tested for immunity to infection with tubercle cultures.

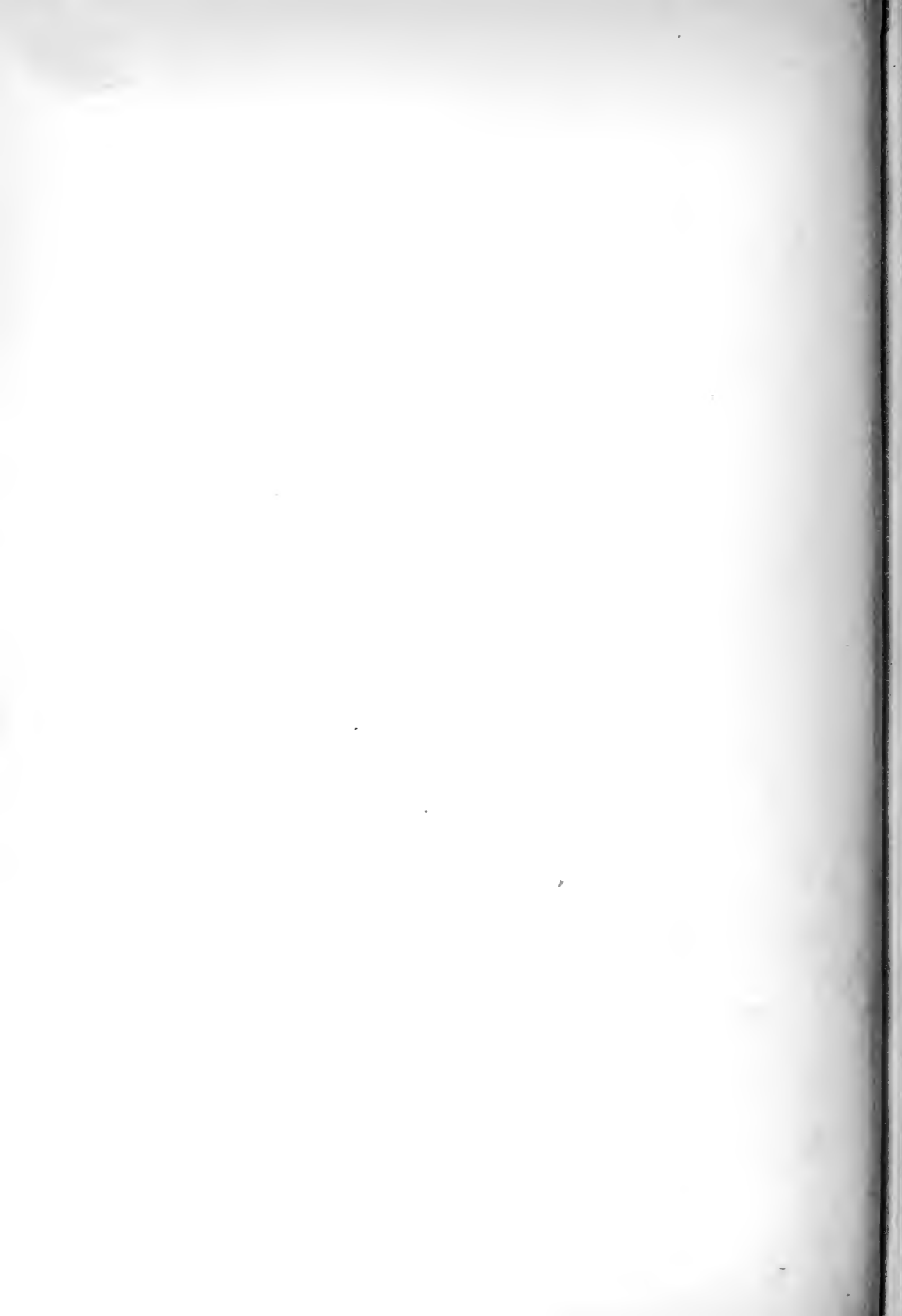
*Typhoid.*—The indications of hypersusceptibility induced by two injections of typhoid extract manifest themselves by rapid respirations; most of the pigs lie down on their sides. The symptoms presented by this series of pigs were mild.

Nine days following the second injection of the extract five pigs, which had received

10 c.c. of the typhoid extract at the second injection, resisted a large dose of a virulent typhoid culture. Two controls died in 18 hours. One or two of the pigs which had received the extract were slightly sick the following day, but the next day had fully recovered and have remained so. A definite immunity was, therefore, conferred by the two injections of extract from the typhoid bacillus.

These results strengthen our belief that the phenomenon of hypersusceptibility has a practical significance in the prevention and cure of certain infectious processes. It gives a possible explanation to the period of incubation of some of the communicable diseases. Is it a coincidence that the period of incubation of a number of infectious diseases is about 10 to 14 days, which corresponds significantly with the time required to sensitive animals with a strange proteid? In certain infectious diseases with short periods of incubation, such as pneumonia, the crisis which commonly appears about the tenth day may find a somewhat similar explanation. It is evident that disease processes produced by soluble toxins, such as diphtheria and tetanus, do not belong to the category now under consideration.

The phenomenon of hypersusceptibility has been produced in the guinea-pig by extracts obtained from the colon bacillus, yeast, hay bacillus, anthrax, tubercle bacillus, and the typhoid bacillus. The hypersusceptibility produced by the colon and typhoid bacillus was followed by a definite immunity to the corresponding infection. In the case of anthrax, however, immunity does not follow hypersusceptibility to the anthrax proteid. We are, therefore, not dealing with a general law applicable to all infections, but with certain limitations as in the case of antitoxic immunity.



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## THE FREQUENCY OF TUBERCLE BACILLI IN THE MARKET MILK OF THE CITY OF WASHINGTON, D. C.\*

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### INTRODUCTION.

NUMEROUS investigators in recent years have shown the infectiousness for animals of milk containing tubercle bacilli. Whether the milk from animals with tuberculosis but with healthy udders contains tubercle bacilli is not definitely settled. Many prominent scientists seem to have known that at times the milk from such animals does contain tubercle bacilli virulent for laboratory animals, but in view of recent work there may be some doubt as to whether the bacilli really passed through the udder or gained access to the milk from contamination with feces containing tubercle bacilli.

Schroeder and Cotton<sup>1</sup> have recently shown that cows so slightly affected with tuberculosis as only to be discoverable by the tuberculin reaction pass virulent bacilli in their feces. Many believe that milk from a tuberculous cow with unaffected udder is free from infection

\* Received for publication February 4, 1908.

<sup>1</sup> *Bull. No. 99*, Bureau of Animal Industry, 1907.

and becomes infected from the feces of the animal or from its environment. This observation is of the very greatest importance, and if confirmed shows more than ever that the greatest care is necessary in guarding milk from contamination from the time it is drawn until it is consumed.

The milk supply of many of the cities of Europe and England has been examined for tubercle bacilli. Most observers have used the animal test, i. e., they have injected various amounts into guinea-pigs or rabbits. The percentage of samples showing tubercle bacilli has varied between very wide limits, no doubt dependent upon the difference in the number of tuberculous cows in the herds supplying milk to the different cities and on differences in technic. Some observers have found that when a number of animals are inoculated with the same samples of milk only one, perhaps, will develop tuberculosis. Some centrifugalized the milk and gave sediment alone, while others gave sediment and cream.

I will not enter into the question as to whether the tubercle bacilli found in milk are virulent for man, but give my results only as determining whether the market milk of the city of Washington contains tubercle bacilli virulent for guinea-pigs.

#### THE NUMBER OF TUBERCULOUS COWS IN THE DAIRIES SUPPLYING WASHINGTON, D. C.

A letter was addressed to Dr. W. C. Woodward, health officer, Washington, D. C., and to the Agricultural Department requesting data as to the number of cows in dairies supplying milk to the city of Washington, which had responded to the tuberculin test. Dr. J. R. Mohler stated in October, 1907, that of 1,147 recently tested cows supplying milk to the city of Washington, 214 or 18.6 per cent, responded to the tuberculin test. He stated that he did not consider this a fair estimate of the extent of tuberculosis in the dairy herds of this vicinity as the tests were being applied only to those herds which had recently been cleansed by private tests or appeared so healthy that their owners had no fear of having them tested.

I am informed by the District Health Department that 1,059 cows from 51 herds in Virginia, Maryland, and the District of Columbia supplying milk to the city of Washington were tested for their reaction



to tuberculin; of this number 160, or 15.1 per cent of the total number of cows tested, responded to the tuberculin test.

Of course the figures above furnished by the Department of Agriculture and the District Health Department do not give a fair idea of the prevalence of tuberculosis in the herds supplying milk to Washington, as only the owners of those herds who had reason to think that their cows were free from tuberculosis permitted the test to be made. If the test had been applied to all the cows supplying milk to the District I have no doubt that the percentage would be very much higher than the figures above would seem to indicate.

The following figures by Salmon<sup>1</sup> show the number and percentage of cattle carcasses condemned for tuberculosis during the years 1901-5 in the meat-inspection service of the Bureau of Animal Industry:

TABLE 1.

Year	Number Carcasses Examined	Percentage of Carcasses Condemned
1901.....	5,219,149	0.10
1902.....	5,559,969	0.14
1903.....	6,134,410	0.14
1904.....	6,359,011	0.16
1905.....	6,096,597	0.18

This does not show the total number of animals affected with tuberculosis, for in many cases only a part of the carcass was condemned, and probably many had the disease so slightly that the entire carcass was passed as fit for food. The following table, also taken from Salmon's article showing the results of the tuberculin test of cattle in some states, is of value in showing the wide distribution of cattle tuberculosis. It must be remembered that most of the herds tested were suspected herds which may account for the very high percentages found. (Table 2.)

The results of some of the earlier workers are open to criticism in view of Rabinowitch's discovery of an acid-fast bacillus in butter morphologically similar to the tubercle bacillus. If guinea-pigs are inoculated with milk or butter containing the acid-fast butter bacillus they may often die, and will present lesions to the naked eye very similar to those produced by the tubercle bacillus.

<sup>1</sup> *Bull. No. 38*, Bureau of Animal Industry, 1906.

TABLE 2.  
RESULTS OF THE TUBERCULIN TESTS OF CATTLE IN VARIOUS STATES.

State	Number Tested	Number Tuberculous	Percentage Tuberculous
Vermont.....	60,000	2,300	3.9
Massachusetts.....	24,685	12,443	50.0
Massachusetts, entire herds.....	4,093	1,080	26.4
Connecticut.....	6,300	.....	14.2
New York, 1804.....	947	66	6.9
New York, 1897-98.....	1,200	163	18.4
Pennsylvania.....	34,000	4,800	14.1
New Jersey.....	2,500	.....	21.4
Illinois, 1897-98.....	929	.....	12.0
Illinois, 1899.....	3,655	560	15.3
Michigan.....	.....	.....	13.0
Minnesota.....	3,430	.....	11.1
Iowa.....	873	122	13.8
Wisconsin:			
Experiment station tests:			
Suspected herds.....	323	115	35.6
Non-suspected herds.....	935	84	9.0
State veterinarian's tests:			
Suspected herds.....	588	191	32.5
Tests of local veterinarians under state veterinarian on cattle intended for shipment to states requiring tuberculin certificate.....	3,421	76	2.2

#### COLLECTION OF SAMPLES AND TECHNIC.

The samples of milk were all collected and brought to the Hygienic Laboratory by an inspector of the Health Department of the District of Columbia. Usually a pint bottle, though sometimes a quart, with the paper cap untampered with was obtained either from the dairy or delivery wagon; the bottle was at once placed on ice by the collector and usually reached the laboratory in about one hour after collection. A few samples were obtained from some of the hospitals and charitable institutions of the District. The milk and cream were well mixed by vigorously shaking the bottle. The sample for plating was taken out with a sterile pipette and then 50 c.c. of the mixed milk was put into a large sterile centrifuge flask; to the 50 c.c. of milk 100 c.c. of sterile water was added; the flask was then put into the centrifuge machine and centrifugated for one hour at about 2,000 revolutions per minute. The milk was diluted with twice its volume of water with the idea that it would decrease the specific gravity of the milk and so permit of the easier sedimentation of the tubercle bacilli. Usually only one animal was inoculated from each sample, though in some cases two animals were used. Guinea-pigs, largely those raised in the laboratory, of as uniform weight as obtainable, were inoculated with 5 c.c. of the sediment of this centrifugalized mixture of milk and water; the inoculation was made subcutaneously in the belly wall. For each

guinea-pig a different syringe was used. All of the guinea-pigs (usually eight, the number of daily samples) inoculated on the same day were kept in the same cage; those that remained healthy being controls on the environment, etc. The guinea-pigs were examined for enlarged glands after about four weeks, and those with enlarged glands were separated from the others so as to avoid the danger of infecting their companions if the glands broke down.

Many of the animals inoculated died from acute infections with the millions of other bacteria in the milk. Autopsies were made of all the animals that died, but no attempt was made to determine the causal organisms other than the tubercle bacillus.

Those guinea pigs that did not die in at least two months were chloroformed after having been tested by tuberculin, and careful autopsies were made on each animal. Smears, cultures, and sections were made from the various organs of the animals that showed any change from the normal. The smears were stained with carbol-fuchsin and examined for acid-fast bacilli. Cultures were made on glycerinized potato and glycerin-agar; in no instance did any of the cultures show a quick-growing acid-fast organism resembling in any way Rabinowitch's butter bacillus. The sections were stained with carbol-fuchsin for tubercle bacilli and also with hemalum and eosine for histological appearances. The above details were carried out with few exceptions in all of the animals that gave a positive result.

It occurred to me that those animals having tuberculosis might be differentiated from those having other infections by giving all of the guinea-pigs alive at the end of two months a sufficient dose of tuberculin to cause the death of the tuberculous animal in less than 24 hours. Several preliminary tests on known tubercular animals showed that 2 c.c. of crude tuberculin given subcutaneously would almost invariably cause the death of such a guinea-pig in from 6 to 18 hours. As high as 7 c.c. of the same tuberculin given to a healthy pig caused only a temporary discomfort passing off in a few hours. A rather hasty search of the literature failed to show that this idea of giving an amount of tuberculin sufficient to cause the death of a tubercular animal as a means of differentiating true tuberculosis from infection with other acid-fast organisms had ever been used by previous workers. The febrile reaction in a sick guinea-pig on account of

the great variation in the temperature of the animal from handling, etc., is too variable a factor, and a more definite reaction, such as the death of the animal, is necessary. The technic was as follows: All of the animals, in lots of about 30, were given early in the morning

TABLE 3.

Dairy	No. of Samples	No. of Samples Lost by Acute Death of Guinea-Pig	No. of Samples Remaining	No. of Samples Positive for Tuberculosis	Dairy	No. of Samples	No. of Samples Lost by Acute Death of Guinea-Pig	No. of Samples Remaining	No. of Samples Positive for Tuberculosis
1.....	3	2	1	0	Brought For'd	144	24	120	6
2.....	3	0	3	0	55.....	3	1	2	1
3.....	1	0	1	0	56.....	2	0	2	0
4.....	2	0	2	0	57.....	1	0	1	0
5.....	2	1	1	0	58.....	2	0	2	0
6.....	3	0	3	0	59.....	1	0	1	0
7.....	1	0	1	0	60.....	1	0	1	0
8.....	2	0	2	0	61.....	2	0	2	0
9.....	4	1	3	0	62.....	1	0	1	1
10.....	2	0	2	0	63.....	1	0	1	0
11.....	8	2	6	0	64.....	2	0	2	0
12.....	1	1	0	0	65.....	2	1	1	1
13.....	1	0	1	0	66.....	2	0	2	0
14.....	1	0	1	0	67.....	1	0	1	0
15.....	2	1	1	0	68.....	3	1	2	0
16.....	1	0	1	1	69.....	4	1	3	0
17.....	1	0	1	0	70.....	3	1	2	0
18.....	3	0	3	1	71.....	1	0	1	0
19.....	4	1	3	0	72.....	2	0	2	2
20.....	5	0	5	0	73.....	5	1	4	0
21.....	5	1	4	0	74.....	3	1	2	0
22.....	3	1	2	0	75.....	2	0	2	0
23.....	1	0	1	0	76.....	2	0	2	0
24.....	5	1	4	0	77.....	2	1	1	0
25.....	3	0	3	0	78.....	1	1	1	0
26.....	1	0	1	0	79.....	1	0	1	0
27.....	4	1	3	0	80.....	2	0	2	0
28.....	4	1	3	0	81.....	6	1	5	0
29.....	3	0	3	0	82.....	1	2	0	0
30.....	2	0	2	0	83.....	1	0	1	0
31.....	1	0	1	0	84.....	6	1	5	3
32.....	3	1	2	0	85.....	2	0	2	0
33.....	4	2	2	1	86.....	3	0	3	1
34.....	3	0	3	0	87.....	2	0	2	0
35.....	2	0	2	0	88.....	2	0	2	0
36.....	1	0	1	0	89.....	1	0	1	0
37.....	1	0	1	0	90.....	1	1	0	0
38.....	4	1	3	0	91.....	2	0	2	0
39.....	4	1	3	0	92.....	3	1	2	0
40.....	2	0	2	0	93.....	3	0	3	0
41.....	5	1	4	0	94.....	1	0	1	0
42.....	1	0	1	0	95.....	8	3	5	0
43.....	3	0	3	0	96.....	3	0	3	0
44.....	5	0	5	0	97.....	2	0	2	0
45.....	4	1	3	2	98.....	4	1	3	0
46.....	2	0	2	1	99.....	3	2	1	0
47.....	2	1	1	0	100.....	3	1	2	0
48.....	1	0	1	0	101.....	1	0	1	0
49.....	3	0	3	0	102.....	4	1	3	0
50.....	2	0	2	0	103.....	4	1	3	1
51.....	2	0	2	0	104.....	4	2	2	0
52.....	2	1	1	0					
53.....	4	1	3	0	Totals.....	272	49	223	15
54.....	2	0	2	0			18%	82%	6.72%
Carried For'd...	144	24	120	6					

2 c.c. of the tuberculin subcutaneously; they were closely watched, and as soon as an animal appeared sick it was placed aside; as soon after death as possible the animal was autopsied; smears, cultures, and sections were made. Of all the guinea-pigs (about 250) that received the tuberculin, *no animal died that did not have tuberculosis*. Two or three that had slight lesions did not die, but became sick. It was noted that all of the animals died whose lesions had caseated. The reaction, I think, was of distinct service in eliminating infections with other acid-fast organisms. The suggestion is made that with some modification the procedure may have a distinct place as an aid in differentiating true tuberculosis from infections with other acid-fast organisms which produce tubercular-like lesions.

Samples of milk were examined from 104 different dairies; 10 samples from seven hospitals and asylums are also included in this list, being charged also to the dairy supplying the milk.

It is interesting to note that where two guinea-pigs were inoculated with the same samples of milk, in two instances both animals showed tuberculosis, and in two instances only one was positive.

Table 3 gives a summary of the protocols. It shows the laboratory number of the dairy, number of samples from each dairy, number of samples lost by the animal dying in less than three weeks of other infections, number of samples remaining for observation, and total number of samples for each dairy positive for tuberculosis.

The same details are shown in Table 4 for the milk collected from the charitable institutions.

TABLE 4.

Hospital	Number of Samples	Number of Samples Lost by Acute Death of Guinea-Pig	Number of Samples Remaining	Number of Samples Positive for Tuberculosis	Remarks
Providence.....	2	0	2	0	(a) Both pigs from sample positive
Children's.....	1	0	1	0	
Georgetown.....	1	0	1	0	
Garfield.....	2	0	2	0	
Sibley.....	1	0	1	0	
Orphan Asylum.....	1	0	1	1 (a)	
Columbia.....	2	1	1	0	
Totals.....	10	1	9	1	
		10%	90%	11.1%	

## SUMMARY.

It will be seen from the tables that of 272 samples of milk, 49 or 18 per cent of the samples were lost by the animal dying in less than three weeks and before sufficient time had elapsed for it to develop tuberculosis. Attention is invited to the fact that the milk from some of the dairies killed acutely a high percentage of all of the animals to which it was given.

Of the 272 samples 223 or 82 per cent remained for study.

Of the 223 that remained, 15 or 6.72 per cent contained sufficient tubercle bacilli to cause typical tuberculosis in the inoculated animals.

Of the samples of milk from 104 dairies two were lost by acute death of the animals, leaving 102; the milk from 11 of these 102 dairies contained tubercle bacilli; this gives a percentage of 10.7 of the dairies examined showing tubercle bacilli in the milk supplied to their customers.

Ten samples of milk were obtained from seven charitable institutions of the District; of these ten samples one was lost by the acute death of the animal, leaving nine samples from six institutions for study. The sample from one institution caused tuberculosis in both guinea-pigs in which it was inoculated.

These results, showing that approximately 11 per cent of the dairies whose milk was examined contained tubercle bacilli virulent for guinea-pigs, do not, however, give a fair idea of the frequency of tubercle bacilli in the market milk of the city of Washington. Attention has already been called to the fact that when two animals were inoculated with the same sample both did not always develop tuberculosis; this might indicate that the bacilli are so few in the amount inoculated that one of the animals by being a little more resistant was able to overcome the infection. The amount inoculated, less than 2 c.c. of milk, is a very small portion of a pint bottle. The creamy layer was not inoculated, and other workers have shown that tubercle bacilli are more frequent in this than in the bottom milk; it is very probable that if more animals had been inoculated with the same sample and both cream and sediment had been used, the percentage of positive results would have been very much higher. The results, however, as they were found, are sufficiently high to emphasize the great necessity for the enactment and rigorous enforcement of a law

requiring that all cows supplying milk to the District be tuberculin tested and free from tuberculosis. This test, which is now universally recognized as a means of determining whether or not an animal has tuberculosis, should be made by a competent veterinarian and those animals that respond should be disposed of in some way so that their milk may no longer be a source of danger to the community.

NOTE.—For a review of the literature on tubercle bacilli in market milk and for details of the autopsies of my work, see article in *Bull. 41, Hygienic Laboratory, Public Health and Marine-Hospital Service*, "Milk and Its Relation to the Public Health," by various authors.

## THE PATHOLOGY OF AMANITA PHALLOIDES INTOXICATION.\*

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UP to the time of Kobert's publications<sup>1</sup> concerning the active principle of *Amanita phalloides*, little was positively known in regard to the poisons of this fungus, although Boudier,<sup>2</sup> Letellier and Speneux,<sup>3</sup> and Oré<sup>4</sup> had each of them made a chemical analysis of the plant and had extracted certain principles to which the names bulbosin, amanitin, and phalloidin had been respectively given. The ground for criticism of the previous work lay in the imperfect identification of the specimens used by the early investigators, and in a possible admixture of *Amanita muscaria* from which Schmiedeberg and Koppe<sup>5</sup> had obtained the alkaloid muscarin. Kobert's studies in 1891 demonstrated that aqueous and saline extracts of fungi, identified beyond peradventure as *Amanita phalloides* and as the fungus known to be the cause of nearly all the fatal cases of mushroom intoxication, contained a powerful hemolytic substance, dissolving a great variety of corpuscles, his dried material acting upon ox blood, for instance, in a dilution of 1 : 125,000. The discovery of the hemolysin in these fungi was a very great advance in the knowledge of poisonous plants and to its presence in *Amanita phalloides* Kobert naturally attributed the toxic action of the fungus on man. This hemolytic substance, to which Kobert gave the name phallin, was highly unstable, easily destroyed by acids, alcohol, etc., and was rendered inactive by exposure to a temperature of 70° C. He believed it to be a proteid belonging to a class of poisonous substances characterized as "toxalbumins," in which group he placed also the hemolytic poison of spiders (Spinnengift). How such a labile substance,

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<sup>1</sup> St. Petersb. med. W'chenschr., 1891, 16, pp. 463, 471.

<sup>2</sup> Des champignons au point de vue de leurs caractères usuels, chimiques et toxicologiques, Paris, 1866.

<sup>3</sup> Ann. d'hyg. publ., 1867, 27-28, p. 71.

<sup>4</sup> Bull. Acad. de méd., 1877, 13, p. 350; Arch. de phys. norm. et path., 1877, 2, p. 275.

<sup>5</sup> Das Muskarin, Leipzig, 1869.



so susceptible to acid and heat as phallin, could be the active principle in poisoning where cooked fungi were taken into the stomach was never satisfactorily explained by Kobert, nor could all the lesions found in fatal cases in man be justly referred to the action of a blood-laking agent alone.

Nevertheless, despite the objections of such excellent authorities as Kunkel,<sup>1</sup> Bourquelot,<sup>2</sup> and others to the complete acceptance of phallin as the active principle of *Amanita phalloides*, Kobert's observations were really the first of any value in this subject and his views were therefore adopted by the majority of mycologists and found their way into all the standard textbooks. In 1899 Kobert<sup>3</sup> published a second communication upon the poisons of the deadly *Amanita* in which he stated that later investigations of the plant failed to reveal the constant presence of phallin, and that in addition, the alcoholic extract of the fungi contained a highly poisonous non-hemolytic substance fatal to small animals in minimal doses but not producing any fatty degeneration of the organs, the lesion which is accepted by all writers on this subject, as the most characteristic change in man. This second substance Kobert stated to be an alkaloid, without, however, specifying any alkaloidal reactions to which it responded. The failure of this substance to produce fatty degeneration precluded, according to Kobert, any possibility of its being the active principle. In 1906<sup>4</sup> we were able to confirm Kobert's observations upon the hemolytic activity of aqueous and saline extracts of the fungus in question and later<sup>5</sup> pointed out the fact that heated extracts, no longer hemolytic, were still powerfully toxic to small animals. The fungus must therefore contain another poison, heat-resistant in character, in addition to the blood-laking substance described by Kobert. To this second poison we gave the name *Amanita-toxin*.

Subsequently, in a chemical investigation of the fungus in the Pharmacological Laboratory, made by Dr. Abel and myself,<sup>6</sup> it was

<sup>1</sup> *Handbuch der Toxikologie*, Jena, 1901, 2, p. 1048.

<sup>2</sup> Article on "Champignons" in Richet's *Dict. de phys.*, Paris, 1898, 3, p. 271.

<sup>3</sup> *Sitzungsb. d. Naturf. Gesellsch.*, 1899, p. 26; Anhang to the *Arch. d. Ver. d. Freund. d. Naturg.*, 1899, 53, zweite Abtheilung.

<sup>4</sup> Ford, *Jour. Infect. Dis.*, 1906, 3, p. 191.

<sup>5</sup> Ford, *Jour. Exper. Med.*, 1906, 8, p. 437.

<sup>6</sup> *Jour. Biol. Chem.*, 1907, 2, p. 273.

shown that on the addition of alcohol to aqueous extracts the hemolysin is precipitated, while the alcohol-soluble fraction contains a substance extremely poisonous to both rabbits and guinea-pigs, identical in its action with the *Amanita*-toxin already described. This *Amanita*-toxin we suspect may be the same poison as described by Kobert in his second communication and characterized by him as an alkaloid. It was also shown by us that the hemolysin in *Amanita phalloides* is not a toxalbumin, as stated by Kobert, since all proteid may be removed from it by the use of freshly prepared metaphosphoric acid and by uranyl acetate without appreciable impairment of its hemolytic activity. For the present, at least, we believe that this *Amanita*-hemolysin, as we shall call it, must be classed at a glucoside, inasmuch as our purest proteid-free hemolytic preparations always reduce Fehling's solution after hydrolysis with mineral acids, and give characteristic tests for pentoses. The extreme lability of this substance and its sensitiveness to the action of heat and acids at once raises the question of its playing any rôle in human intoxications. The observations of Dr. Schlesinger and myself<sup>1</sup> finally upon the chemical properties of *Amanita*-toxin indicated that this substance is not a proteid or an alkaloid, but either an indol or pyrrol derivative or an aromatic phenol so combined with an amine group that it readily forms an indol or pyrrol ring on fusion. In the last edition of his textbook,<sup>2</sup> Kobert's views have undergone another modification. In this publication he assigns to the fungus three separate poisonous substances, the hemolytic principle, phallin, the alcohol-soluble alkaloid not producing fatty degeneration, and finally a "toxalbumin" present in the alcoholic precipitate of the aqueous extract, allied chemically to the substances thujon and pulegon, and capable like them of causing fatty changes in the internal organs. As far as can be judged from this latest communication, Kobert presents no experimental evidence as to the presence of this third poison in *Amanita phalloides*, assuming it to be present there in order to explain the fatty degeneration seen in fatal cases in man. It therefore becomes necessary to consider the subject more fully from the pathological standpoint, to determine in how

<sup>1</sup> *Jour. Biol. Chem.*, 1907, 3, p. 279.

<sup>2</sup> *Lehrbuch der Intoxikationen*, Stuttgart, zweite Auflage, 1906, 2, p. 614.

far the lesions found in man can be explained by the action of these poisons already demonstrated in the plant, and to inquire into the necessity of hypothecating any other toxic substance.

#### LESIONS FOUND IN AMANITA PHALLOIDES INTOXICATION IN MAN.

The principal pathological changes in fatal cases of this intoxication were described originally by Maschka<sup>1</sup> over a half-century ago. From a series of seven autopsies he summarized the lesions as follows: (1) Lack of post-mortem rigidity; (2) Widening of the pupils; (3) Failure of the blood to coagulate and a cherry red color; (4) Ecchymoses and hemorrhages in the serous membranes and parenchymatous organs; (5) Dilatation of the bladder with urine. In addition Maschka observed a fatty degeneration of the internal organs, a change not noted by the earlier observers, but failed to regard this lesion with the attention it deserved.

Carayon<sup>2</sup> in 1873, in autopsies upon soldiers who died three days after eating cooked *Amanita phalloides*, noted an inflammation of the walls of the stomach and intestine, a congestion of the liver and kidney, a dark red and fluid condition of the blood, and in one case, a congestion of the meninges, thus confirming Maschka's observations in part. A little later Chouet and Pelissié<sup>3</sup> studied the lesions in two individuals dead in 48 and in 60 hours from the time of ingestion of the fungi. They found the contents of the stomach and intestine bloody, with ecchymoses in the gastric mucosa and in the liver. The meninges also were hyperemic and the pia-mater showed ecchymoses. Possibly the most careful microscopic examination of the organs in this condition was made by Sahli<sup>4</sup> in two cases. The gross lesions were similar to those described by Maschka, and included subpleural and intrapulmonary hemorrhages and a general atrophy of the panniculus adiposus. In addition Sahli found a fatty degeneration of the liver and kidney, the heart, and the diaphragm, as well as of the voluntary muscles, such as the pectorals, deltoid, abdominal, and the tongue. The amount of fat in the liver was so great as to remind him of acute phosphorous poisoning, with

<sup>1</sup> *Vrtljschr. f. d. prakt. Heilk.*, 1855, 46, p. 137.

<sup>2</sup> *Gaz. d. hôp.*, 1873, 46, p. 1115.

<sup>3</sup> *Gaz. hebdomadaire de méd.*, 1880, 2d s., 17, p. 68.

<sup>4</sup> Studer, Sahli, und Schärer, *Mitth. der naturforsch. Gesellsch.*, 1885, p. 81.

which phalloides intoxication has ever since been compared. In the alimentary tract the mucosa of the stomach and intestine were much congested, the Peyers patches and solitary follicles swollen. In 1886 Handford<sup>1</sup> in an autopsy on a man dead three days after being poisoned, found ecchymoses on the pleural and pericardial surfaces, a congestion of the mucous membrane of the stomach, with capillary hemorrhages, and excoriations (ulcers), and a general congestion of the intestine. The kidney was anemic and the liver fatty.

Tappeiner<sup>2</sup> subsequently had the opportunity of carefully studying the bodies of two children dead from eating *Amanita phalloides*. In one case there were a number of small punctiform hemorrhages beneath the skin and in both cases the stomach and intestines showed the characteristic injection of the mucous membrane with small hemorrhages, and a swelling of the solitary follicles, the Peyers patches, and the mesenteric lymphatics. Minute hemorrhages were also visible on the surface of the spleen, the kidney, and the liver, and on section of these organs similar changes were apparent. In one of the cases in which jaundice was present during life, the liver was yellowish green in color and brittle in consistency. On microscopic examination, the liver and kidney revealed fatty degeneration and infiltration of the cells, with infarcts, like the changes seen in phosphorous poisoning, while similar infarcts in the heart muscle showed evidences of blood destruction. The heart muscle also was fatty. The percentage of fat in the liver was estimated in these two cases by Tappeiner, who found 53.6 per cent in one case and 68.9 per cent in the other. The fat content of the liver under normal circumstances varies from 8 to 25 per cent (Perls), and in these cases the percentage of fat is thus equal to that found in phosphorous poisoning, usually from 50 to 70. This percentage of fat obtained by Tappeiner was confirmed by Thiemich<sup>3</sup> who estimated the amount in the liver in one case at from 69.1 to 69.3 per cent and in another at from 73.1 to 73.5 per cent.

The lack of post-mortem rigidity noted by Maschka and by Sahli and the widening of the pupils mentioned by Maschka were confirmed

<sup>1</sup> *Lancet*, 1886, 2, p. 1018.

<sup>2</sup> *Munch. med. Wchnschr.*, 1895, 42, p. 133

<sup>3</sup> *Deutsche med. Wchnschr.*, 1898, 24, p. 760.

by Moers,<sup>1</sup> whose three cases were especially marked by the extent and distribution of the hemorrhages. These were present in the stomach and intestine, on the surface and in the substance of the liver and kidney, in the heart, the pericardium, the external coat of the aorta, and in two cases in the ovary, and in the brain. In these cases the various organs were tested for arsenic, phosphorous, and muscarin, in the absence of which substances the lesions found could only be attributed to the poisons of the fungus itself. Finally Plowwright<sup>2</sup> has recently reported for the second time an autopsy upon a boy of 12 years, who died five days after eating a third of the pileus of an uncooked specimen. In this case the gastric mucosa was much injected and softened, there were gangrenous spots on the mucous membrane of the intestine, and a general peritonitis. In another autopsy reported at the same time, the mucous membrane of the colon was studded with pearly white tubercles which turned out to be the much enlarged solitary glands.

From these various observations, it is evident that the most important lesions in man consist of the intestinal ulceration, the swelling of the lymphatic tissue, the general congestion of all the organs, the widespread hemorrhages, the necrosis and the fatty degeneration, especially marked in the liver and kidney. This picture is not one of a hemolytic intoxication, such as is that produced by the ingestion of *Helvella esculenta*, whose active principle, helvellic acid, is a blood-laking poison resistant to the action of heat and the digestive juices. The two most important signs of blood destruction, hemoglobinuria and increased pigmentation, particularly of the spleen, while not entirely lacking, are so little in evidence as to escape mention. The evidence at hand points rather to the action of a profound cellular or protoplasmic poison, acting upon the cells of the parenchymatous organs, and resulting in an extensive deposition of fat, and also causing profuse hemorrhages, probably by a similar destructive action upon the cells of the capillary endothelium. The clinical data available lead one to a like conclusion, since vomiting and diarrhea, with exhausting hemorrhage from the mucous membranes and profound prostration are among the early symptoms, followed in the severe cases by

<sup>1</sup> *Ztschr. f. med. Beamt.*, 1903, 16, p. 412.

<sup>2</sup> *Brit. Med. Jour.*, 1905, 2, p. 541.

rapid loss of weight, jaundice, coma, and death. No mention is made of the presence of blood pigment in the urine, and in one case of which I have personal knowledge, the urine was free from color. While the pathological changes, as described, and the clinical symptoms both tend to rule out the action of the hemolysin in alimentary intoxications, this point cannot be definitely settled until a more careful study can be made of cases in man, with the especial end in view of determining the extent to which blood destruction has taken place, and how far it can be deemed responsible for the death of the patient. Some of the lesions mentioned, such as the fluid condition of the blood, seem to indicate, moreover, a certain amount of blood destruction. Since the characteristic lesions are the intestinal ulcers, the fatty degeneration of the organs, and the hemorrhages, it is necessary to determine whether these can be produced experimentally in animals, and to which fraction of the fungus they can in all probability be assigned. It is of especial importance to determine which portion of the fungus can produce fatty degeneration, since Kobert has stated that his alcohol-soluble alkaloid is incapable of causing such a change and has hypothesized a third poison, a toxalbumin like thujon or pulegon, in order to explain this excessive fat production in man.

#### LESIONS PRODUCED BY THE WHOLE EXTRACT

The action of the aqueous or saline extract of *Amanita phalloides* is the same upon both rabbits and guinea-pigs, the amount of the poison necessary for a fatal dose depending largely upon the weight of the animal selected. The animals usually die after the lapse of four to six days, but may succumb to large doses within 24 or 48 hours. At autopsy an extensive gelatinous edema is found in the subcutaneous tissues at the site of inoculation, the edematous tissue exuding a thin reddish fluid on pressure. Minute hemorrhagic areas are visible everywhere in the neighboring fascia and muscular tissue, while the adjacent lymphatic glands are swollen and hemorrhagic. On opening the abdominal cavity, the blood vessels are found much injected, but the most noticeable phenomenon is the presence of the widespread hemorrhages. These vary in size from small ecchymoses on the surface of the liver and kidney to considerable collections of blood between the layers of the peritoncum. Both liver and kidney

are congested, showing on section a general dilatation of the blood vessels with occasional minute areas of hemorrhage. The adrenals exhibit this condition of congestion and hemorrhage to a marked degree, while the lymphatic glands in the abdominal cavity show similar changes. In female animals, both ovaries and uterus may show areas of hemorrhage, and in one case—a pregnant rabbit—there was considerable free blood in the amniotic sac. The bladder is usually filled with urine which is deeply blood stained. On centrifugation no intact blood corpuscles are present, the urine retaining its reddish hue, a result which points to a true hemoglobinuria. In the pleural cavity, hemorrhages are found between the folds of the pleura and minute ecchymoses on the surface of the lung. Rarely more extensive hemorrhages are produced, an entire lobe of the lung or a considerable portion of it being occupied by the extravasated blood. The heart is in complete diastole, the blood in the heart and in the larger vessels remaining fluid or partially coagulated. The meninges of both spinal cord and brain show little areas of bleeding and on section of these organs small punctiform hemorrhages can be made out. The contents of the stomach and intestine are often blood stained, minute ulcers in the mucosa of the bowel, with extravasated blood in the base of the ulcers, indicating the source of the hemorrhage.

Microscopic examination of the tissues and organs reveals constant and fairly characteristic changes. The connective and muscular tissue is much swollen, the muscle fibers show hyaline degeneration and extravasated blood corpuscles can be seen near the blood vessels and between the muscle bands. The lymphatic glands, both those from the subcutaneous tissues and those in the abdominal cavity, are congested and hemorrhagic, the lymph cells are necrotic with pyknotic nuclei, and there is a general increase in pigment. In the spleen there is always considerable extravasated blood, but the prominent change is the very great increase of blood pigment, while the cells of the splenic pulp are necrotic with pyknotic nuclei. In the liver in addition to the congestion of the vessels, many blood corpuscles lie free in the inter- and intra-lobular spaces and between the hepatic cells. Rarely a direct connection can be traced between these areas of extravasated blood and some one of the smaller capillaries,

where a break in the lumen of the vessels allows the corpuscles to escape. The nuclei of the capillary endothelium are pyknotic, the liver cells show necrosis, and there is a general increase of pigmentation. In the kidney there is a uniform condition of congestion and hemorrhage, the kidney cells are shrunk from the basement membrane and are the seat of hyaline degeneration. The lungs show extensive vascular dilatation with many free blood corpuscles and an excess of blood pigment. The muscle fibers of the heart exhibit hyaline degeneration, nuclear vacuolation, and at times almost complete destruction leading to the appearance of small areas of focal necrosis. Finally there is in all the organs evidence of fatty degeneration, this fat being widely distributed, especially in the cells of the liver and kidney. The pathological changes found in animals thus inoculated with the whole extract of this fungus consist mainly of intestinal ulcers, hemorrhage, necrosis and fatty degeneration, a laky condition of the blood, hemoglobinurea, and wide deposition of blood pigment pointing to an extensive blood destruction. While evidence of all these changes can be secured in animals dying at various intervals, their extent varies considerably. Thus large doses of the whole extract and doses of freshly prepared material produce a more fluid condition of the blood, more pronounced hemoglobinurea and a greater increase of free blood pigment than do small doses, more nearly approaching a minimum fatal dose, or older extracts. With large doses, a greater amount of poisonous substance is injected than is necessary to kill the animal and the excess of hemolysin can exert its destructive effect upon the corpuscles. With the freshly prepared extracts, the hemolysin is more active and smaller quantities serve for a lethal dose than with the older extracts, where the hemolysin can be shown by test-tube experiment to have deteriorated considerably in strength. Changes due to blood-laking are thus naturally more pronounced. By heating the whole extracts to 65°-70° C. for one-half hour, the hemolysin is destroyed, yet the solutions do not lose their toxicity, owing to the presence of the heat-resistant toxin. By inoculating feeble guinea-pigs, with small doses of the fresh extract an acute death can sometimes be brought about apparently by the action of this toxin, since in these cases but little evidence of intra-corporeal blood-laking can be made out.



It is manifest that when we are employing the whole extract, we are inoculating into the test animals a mixture of poisons, the relative amount of which depends upon the age of the preparation, while the lesions produced in the inoculated animals vary with the toxicity of each ingredient and the extent to which it is present in the dose administered. Let us now try to determine what lesions can be produced in animals by the inoculation of the different poisons of the plant, freed from each other, as far as possible, by chemical means.

#### LESIONS PRODUCED BY THE AMANITA-TOXIN.

The alcoholic extract of *Amanita phalloides*, free from the hemolytic glucoside, is extremely poisonous to both rabbits and guinea-pigs and is identical in its action with extracts of the fungus heated to 70° C. and containing the Amanita-toxin previously described. With large doses the animals die acutely, often within 24 or 48 hours, while with lesser amounts death occurs usually after the lapse of six or eight days. At autopsy, there is little or no change at the site of inoculation, except a slight edema, which may be obviated by careful neutralization of the extract. Rarely there is a considerable injection of the blood vessels at this area and minute points of ecchymoses may be found scattered through the adjoining fascia and muscles. The lymphatic glands sometimes present no change, but are more frequently congested and hemorrhagic. In the abdominal cavity the vascular changes predominate, ecchymoses and punctiform hemorrhages showing on the surface of the internal organs, with extravasated blood in the mesentery. The organs are pale and the liver especially looks fatty. The adrenals are sometimes normal in appearance but frequently show a condition of extreme congestion and hemorrhage. The bladder is filled with urine, always straw colored and free from blood pigment. In the pleural cavity, small flecks of hemorrhage are visible on the surface of the lungs, and rarely large amounts of blood are found between the layers of the pleura. The heart is widely dilated, the blood always firmly clotted, the same condition holding true for the larger vessels. Occasionally small petechiae are seen on the pericardial surfaces. The spinal cord and brain show no especial changes. In the walls of the stomach and intestine are often found minute ulcers, and there is frequently

dried blood in the contents of the bowel. Careful search nearly always reveals the source of this blood.

Microscopically the changes found in the tissues and organs, relate chiefly to necrosis, fatty degeneration and to hemorrhage. The voluntary and cardiac muscles show no particular edema, but many free blood corpuscles outside the smaller vessels and considerable hyaline degeneration. In the lymphatic glands there is a congestion of the vessels and some extravasation of blood, while the lymph cells show necrosis. There is no special increase of pigment. In the spleen the pigment is increased slightly but not markedly, but there is considerable extravasated blood and the cells of the splenic pulp are necrotic. In both liver and kidney there is an extraordinary deposition of fat. This is present in fine granules or in coarse globules within the cells, and so extensive is this deposition that it seems as if almost every cell had undergone this fatty change. In some animals the liver appears almost entirely transformed into fat. Of especial interest is the presence of fat in the cells of the capillary endothelium, suggesting that the various hemorrhages may be due to a rupture of these small vessels consequent upon a fatty degeneration.

Similarly in the kidney, there is an extreme fatty degeneration of the cells of the glomeruli and of the various tubules, as well as in the endothelial lining of the capillaries. In the lungs, the microscopic changes are chiefly vascular, showing a general dilatation of the vessels and an extravasation of the blood. There is no special increase of pigment in this organ. The gross lesions produced by the *Amanita*-toxin represent to only a partial degree, therefore, the lesions produced by the whole extract. There is no edematous swelling at the site of inoculation, and no hemoglobinuria. The hemorrhages are by no means as extensive as after inoculation of the whole extract, but they are constantly present and may occur in any situation. Small ulcers may be present in both stomach and intestine. Microscopically the excessive pigmentation of the spleen is absent and the increase of pigment in the lymph glands and in the lungs is less marked. The necrosis and fatty degeneration are present to a greater degree possibly, but this lesion is more easily brought out in these tissues than in the tissues of animals dead of both toxin and hemolysin, since the greater deposition of pigment

and the more extensive escape of blood corpuscles in the latter case may to a certain extent obscure the fatty change. Moreover, but one poison is here administered and larger amounts of this single poison are necessary for a fatal dose than when a poisonous hemolysin is also present, in consequence of which the lesions due to this substance would naturally predominate. In some cases indeed the fatty degeneration seems to be the only lesion present, the evidence of hemorrhage being very slight. Finally the solutions containing the toxin can be heated to between 70° and 80° C. without losing their activity.

#### LESIONS PRODUCED BY THE AMANITA-HEMOLYSIN.

The Amanita-hemolysin contained in the alcohol precipitate of extracts of the fungi is poisonous to animals but by no means in the same degree as are solutions containing the toxin alone, since the doses required to kill either rabbits or guinea-pigs are much larger. Animals may die acutely in 48 hours from these large doses, but usually death ensues at a later period, possibly after 10 or 12 days. The hemolysin is essentially not so acute a poison as the toxin. At autopsy the most marked lesion is the swelling and edema at the site of inoculation with the production of a large amount of fluid deeply stained by blood pigment. The glandular apparatus shows little change microscopically. In the abdominal cavity there is an exudate of blood-stained fluid with a general congestion of the blood vessels, and frequent hemorrhages. The bladder is full of urine, stained dark red by free blood pigment. The heart is dilated and full of laked and fluid blood which fails to clot on exposure to the air. Similar fluid blood occupies the larger vessels. There is no special lesion in the lungs.

On microscopic examination, the most marked change is the great increase of pigment. The spleen is loaded with this substance which is also much increased in the lymphatic glands and in the liver and lungs.

There is some increase of fat in the various organs but this is not present to any considerable extent. There is some extravasation of blood.

The lesions in animals poisoned with this portion of the fungus,

are due to the blood-laking properties of the hemolysin and the whole picture is one of a hemolytic intoxication. The two most characteristic signs of such an intoxication, hemoglobinurea and pigmentation of the spleen, are present to an extreme degree. If extracts containing the hemolysin alone, free from the toxin, be heated to 75° C. for half an hour they lose their blood-laking properties and then may be given in large quantity to animals without injurious effect. Sometimes a little edema develops at the site of inoculation, but the fluid is not stained with hemoglobin and if the extract be made neutral before administration this edema does not develop. It may be due to the irritating action of some acid present in the original aqueous extract of the plant and precipitated by ethyl alcohol.

#### SUMMARY OF PATHOLOGICAL CHANGES.

The principal lesions found in phalloides intoxications in man can be produced in animals by the administration of alcoholic extracts containing the toxin, freed from the hemolytic glucoside. These lesions are the hemorrhages, the cellular necroses, the fatty degeneration, and the gastric and intestinal ulcers. They may also be produced by aqueous extracts of the plant containing both toxin and hemolysin, but with the whole extract the hemorrhages are far more extensive, and there is in addition a true hemoglobinurea and a great increase in the pigmentation of the organs, especially the spleen. The *Amanita*-hemolysin free from toxin reproduces these latter lesions but not the important lesions seen in man. The toxin alone reproduces none of the phenomena of a hemolytic intoxication. The sensitiveness of the *Amanita*-hemolysin to heat and acids, and the resistance of the *Amanita*-toxin to these agencies, would indicate that the hemolysin plays but an unimportant rôle in alimentary intoxications where the cooked fungi are introduced into the stomach, in conformity with which is the fact that in man the lesions generally described point to the action of a protoplasmic poison, and not to a hemolytic agent. The action of the hemolysin in man, cannot be entirely ruled out on pathological grounds, since our knowledge of the pathology of human intoxications is far too meager to permit positive conclusions as to the character of the microscopic changes. It has also been shown that hemolytic substances are present in two

species of fungi usually believed to be edible, *Amanita solitaria*, and *Amanita rubescens*,<sup>1</sup> and that certain specimens of *Amanita phalloides* typical in other respects are devoid of hemolytic activity. In the majority of instances, however, and in all cases where chemical examination of the plant has revealed the presence of a powerful toxin, *Amanita phalloides* contains an abundant and extremely active hemolysin, which is present regardless of the locality from which the fungi are collected. In the ordinary method of cooking fungi for the table this hemolysin may not be entirely destroyed and it may be introduced into the stomach in such large quantities as to escape the action of the gastric and intestinal juices, especially as if these should be deficient in acid or in ferments. Furthermore, in many fatal cases of mushroom intoxication the fungi are eaten improperly cooked and even raw, as for instance by little children playing in the woods. For these reasons it is impossible to eliminate the action of the Amanita-hemolysin in man, although it is evident that the Amanita-toxin plays the more important rôle of the two substances. Just what relation the alcohol-soluble alkaloid described by Kobert bears to our alcohol-soluble Amanita-toxin is not clear since he expressly stated that his substance does not produce any fatty degeneration. The Amanita-toxin, however, produces this fatty change to a marked extent, and is undoubtedly responsible for this lesion in man. On this account there is no need to hypothesize a third poisonous substance, a toxalbumin like thujon and pulegon to explain the fatty degeneration of liver and kidney so characteristic of human intoxications.

#### ALIMENTARY ADMINISTRATION OF AMANITA PHALLOIDES IN RABBITS.

In an attempt to reproduce in animals the conditions of intoxication by this fungus in man, an attempt has been made to poison rabbits through the administration of various extracts of the fungus by mouth. A small rubber tube was passed into the stomach and both hemolysin and toxin given in large quantities. To our great surprise it was found that these animals are absolutely resistant to the action of the poisons of this fungus in this method of administration. Both hemolysin and toxin are quite innocuous. This was of special interest in regard to

<sup>1</sup> Ford, *Jour. Infect. Dis.*, 1907, 4, p. 434.

the toxin which we know is not destroyed by the digestive ferments in man, and on several occasions highly toxic solutions were given without injurious effects. As much as 10 c.c. of a toxin in which  $\frac{1}{4}$  c.c. would kill rabbits on subcutaneous inoculation was administered to a number of rabbits, but there seemed to be no harmful results. Extracts of other *Amanitas*, *Amanita solitaria* and *Amanita rubescens*, containing strong hemolysins were also given to rabbits by mouth but no injurious effect was seen. Whether it is an infallible rule that rabbits can take these poisons into their stomach without disastrous consequences, or the mechanism by means of which the poison is destroyed in their alimentary canal is not known. It would, however, be remarkable in the economy of nature, if such an abundant plant as the *Amanita phalloides* could not be used as food by some animals, and possibly it can be so utilized by the herbivora. We know that both dogs and cats are poisoned by the cooked fungi in the same degree as are human beings.

#### APPLICATION OF THESE FACTS TO SERUM-THERAPY.

In our early work on the production of immunity in animals, by the administration of gradually increasing doses of extracts of the fungus, the claim of Kobert that the hemolysin in this plant is the active principle was believed to have been established. It was not found difficult to produce an active immunity in rabbits in which the animals would withstand the inoculation of two or three fatal doses, and the serum from these animals was highly antihemolytic, in a dilution of 1:1,000, or as high as 1:5,000. When tested upon animals it was also antitoxic to a limited degree, 1 c.c. of the serum neutralizing two or three times a minimum fatal dose. While the strongest serum obtainable neutralized but six or seven times a fatal dose, this serum contained such a powerful antihemolysin that we were led to believe that a serum from large animals more highly immunized might be of therapeutic use. The antihemolytic power of the serum *in vitro* was looked upon as a valuable index in the estimation of its antitoxic power.

In attempting to produce a higher degree of immunity, however, the mortality among the animals was much increased, the cause of this phenomenon not being apparent at that time. A similar experience with larger animals such as goats and horses indicated that there

were other factors to be considered than the hemolytic activity of the extracts and the amount of antihemolysin the animals produced. The most valuable serum obtained was made by Dr. Kinyoun from a horse, at the Mulford Laboratories at Glenolden, Pa. Although the antitoxic power of this serum was so low as to be of little practical value,  $\frac{1}{4}$  c.c. of the serum neutralizing a toxic dose of the fungus for a 500-gram guinea-pig, yet this serum contained a strong and permanent antihemolysin operative in a dilution of 1:1,000, using as an index that quantity of hemolysin which will dissolve 1 c.c. of a 5 per cent blood suspension. It has now been shown by the observations of Kobert and by our own work that the hemolysin in the fungi is accompanied by this powerful toxin, which may play the more important rôle in man. Experiments were therefore instituted to determine the extent to which animals can be immunized to these two substances completely separated by chemical means. With the pure hemolysin freed from toxin, rabbits can easily be immunized. There is at first a slight loss in weight, and some edema at the site of inoculation. The animals soon recover their weight, the subcutaneous edema disappears, and after five or six doses, fairly large quantities of the hemolysin can be administered. The serum from these immunized animals is antihemolytic, a strength of 1:1,000 being obtained after half a dozen doses. With the pure toxin, immunization is attended with a high mortality and the serum produced by these animals successfully immunized has but a limited quantity of antitoxin. Active immunity up to the administration of two or three multiples of a fatal dose is not difficult, but beyond this point the animals die just as they do with the whole extracts. At no time has a stronger serum been obtained with this pure toxin than with the mixture of toxin and hemolysin originally employed. We are therefore confronted with this condition. It is possible to make an antibody for the hemolysin in the fungus and such an antihemolysin would have practical value to just that extent to which the hemolysin acts in man. Although attempts are still being made to prepare an antitoxic serum, for that portion of the fungus which plays the more important rôle in man, the results thus far obtained have not been accompanied with a sufficient degree of success to justify any attempt to make a practical application of our work. The demonstration by Dr. Abel and myself

that the *Amanita*-hemolysin, for which we have repeatedly made an antihemolytic serum, is a glucoside is not without theoretical interest, both in regard to current opinion on this subject and with reference to the possibility of making antibodies to other poisons of a glucosidal nature.

#### CONCLUSIONS.

1. The lesions found in fatal cases of *Amanita phalloides* intoxication in man consist of ulcers in the stomach and intestines, hemorrhages in the serous membranes and parenchymatous organs, necrosis of various cells, and fatty degeneration, especially advanced in the liver and kidney.

2. The lesions produced by the whole extract of the fungus consist of gastric and intestinal ulcers, hemorrhages, necrosis of cells, fatty degeneration, hemoglobinurea, and pigmentation of the spleen and other organs.

3. The lesions produced by the *Amanita*-toxin consist of gastric and intestinal ulcers, hemorrhages, necrosis, and fatty degeneration, and to a certain extent they approximate the lesions seen in man.

4. The *Amanita*-hemolysin acts upon animals by virtue of its blood-laking properties, producing the hemoglobinurea and the pigmentation of the spleen characteristic of hemolytic intoxications.

5. The *Amanita*-toxin is probably the more active principle in alimentary intoxications in man since with it we can produce experimentally the lesions usually found. The action of the hemolysin cannot be ruled out in cases where large quantities of insufficiently cooked fungi are consumed or where the fungus is eaten raw.

6. Inasmuch as the *Amanita*-toxin will produce fatty degenerations in a marked degree, there is no need to assume the presence of a hypothetical toxalbumin like thujon or pulegon in order to explain the fatty character of the lesions.



GONORRHEAL VULVO-VAGINITIS IN CHILDREN.\*  
WITH SPECIAL REFERENCE TO AN EPIDEMIC OCCURRING IN  
SCARLET-FEVER WARDS

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HISTORICAL

THERE is no disease that has had a more varied history or that has passed through stages more confused and contradictory than gonorrhea. Throughout ancient history and up to the end of the Middle Ages it was apparently recognized in its true character as a specific and communicable disease, but the awakening of medical science during the fifteenth century had the curious result of obscuring rather than enlightening this particular field, and gonorrhea came to be hopelessly confounded with the far more striking disease, syphilis. It is interesting to note that one of the earliest attempts at experimental medicine, Hunter's famous inoculation experiments with syphilis, served to strengthen enormously this erroneous conception, for the virus used by Hunter evidently came from a case of mixed infection and both gonorrhea and syphilis resulted from the inoculation. From this time on we find gonorrhea regarded only as a symptom of syphilis, and the subject does not emerge from this stage of confusion until well on into the nineteenth century when, chiefly through the work of Ricord, the two diseases are at last recognized as distinct.<sup>1</sup>

The next stage in the history of gonorrhea is that of controversy over its specificity and infectious character, and is filled with observations and experiments tending to prove that gonorrheal pus is not the same as ordinary pus, and cannot be produced by inoculation from ordinary pus or by ordinary irritants. This point having finally been proved beyond controversy, it remained to discover the specific cause. Neisser's discovery of the gonococcus in 1879 was finally and universally accepted only after Bumm's publication, in

\* Received for publication February 27, 1908.

<sup>1</sup> There is an interesting review of the old literature in Epstein's monograph. See references at end of article.

1884, of his work in successfully growing the gonococcus in pure culture on artificial medium and producing gonorrhea by inoculation experiments in human beings.

Since then the history of gonorrhea has been the history of a disease gradually increasing in importance. One after another serious lesions in various parts of the body and fatal general pyemia have been found to be caused by gonococcal infection. The highly contagious nature of the disease has also come to be recognized and, of late years, its extreme chronicity and obstinacy.

The history of gonorrhea in children has passed through the same stages as that of gonorrhea in adults, only it has always halted behind and often dropped into complete obscurity. Two things have contributed to retard the clearing-up of this subject. First, the apparently insignificant character of the disease in children and, second, the firmly rooted belief still persisting in the popular mind, that gonorrhea can be conveyed by sexual contact only. The first of these, the belief that leucorrhea in childhood is of slight importance, must explain why it is that we find so few references to it in the early literature. Occasionally some unusually observant physician will note the occurrence of a "fluor albus" which has apparently been communicated by a mother to her child, or which has caused a home or asylum epidemic, but such references are very few. The textbooks on children's diseases up to about 1892 made no mention of infectious vulvo-vaginitis.

Later on we find the subject increasing in importance, but not in clearness. Long after the medical world had accepted the distinction between syphilis and gonorrhea, it continued to regard leucorrhea in little girls as a symptom of hereditary syphilis. When it was finally recognized that gonorrheal infection was possible in childhood it was held that sexual contact alone could be responsible for such infection, and the question then became involved in medico-legal complications. If the vaginitis of a little girl were gonorrheal it must mean that criminal assault had taken place; if none had occurred, the vaginitis must be non-gonorrheal. Neisser states that one of the arguments used against the etiological significance of the gonococcus was the occurrence of cases of vaginitis in institutions for little girls, where there was no possibility of venereal infection. This view has held

sway until very recent years and we find traces of it still in the literature. The vulvo-vaginitis of little girls was admitted to be communicable and probably specific, but inasmuch as it was non-venereal it could not be caused by gonorrheal infection. Scrofula, anemia, marasmus, or the acute exanthematous diseases, or various skin infections were usually regarded as causing it, even when it appeared in epidemic form.

The report published by Atkinson in 1879 is always quoted as the earliest detailed account of an epidemic of infectious vulvo-vaginitis and ophthalmia in little girls. It is Atkinson's great merit that he recognized the connection between the stomatitis and ophthalmia, which had appeared sometime before in the institution, and the sudden outbreak of vulvo-vaginitis. The infectious agent he thought had been carried by the children's fingers from eyes and mouth to vulva. Atkinson called the disease "vulvitis due to a specific contagion other than venereal," sharing the view general at that time that gonorrheal infection was necessarily venereal. Even after the discoveries of Neisser and Bumm had made it possible to determine the real nature of any vaginal discharge, we find this same impression prevailing. Thus Leszynsky describes an epidemic of vulvo-vaginitis affecting 212 little girls in a charitable institution. The disease was evidently highly contagious and there were 18 cases in which ophthalmia developed, but Leszynsky found no evidence that it was "specific" and thought that it arose from uncleanness, scrofula, anemia, etc. Bouchut also recognized the contagiousness of leucorrheal discharges in little girls and the occurrence of the disease in epidemics, but he, like Atkinson, assumed a special infectious agent, a "contagion leucorrhéique," which he believed to be transmitted in various ways, among others by the air.

Fraenkel in 1885 was the first to make bacteriological studies of the vaginal discharge of little girls, using the cases which occurred in a hospital epidemic in Hamburg, but although he succeeded in finding in the discharges an organism morphologically identical with the gonococcus of Neisser, he denied its real identity on the ground of its slight virulence. The disease in his youthful cases was not as serious as the gonorrhea of adults, and as further argument, Fraenkel adduces the fact that he failed in his attempt to produce

ophthalmia in three moribund cases by rubbing vaginal pus on the conjunctiva.

The real nature of this affection in children was, however, recognized by other observers, even before Neisser's discovery. As early as 1878 an American physician, John Morris of Baltimore, ventured the assertion that gonorrhea is a frequent disease in little girls, infection being easy in them because of the prominence of the external genitals, and that contagion is often conveyed indirectly by a gonorrheal father or mother and may cause house epidemics.<sup>1</sup> Morris' article was not very widely read and it remained for Pott<sup>2</sup> in 1883 to call general attention to the great frequency of vaginitis in children and to state that this disease is usually gonorrheal and conveyed indirectly. Czéri's excellent article followed within two years, describing an epidemic of 26 cases in 25 of which he succeeded in isolating and cultivating the gonococcus. Czéri is outspoken in his conviction as to the gonorrheal nature of the chronic catarrhal vulvo-vaginitis of childhood. Indeed, he considers that the burden of proof rests upon those who hold that this disease is ever non-gonorrheal. He reviews Fraenkel's work and points out the flaws in the latter's reasoning, especially as to the failure of his inoculation experiments, which, performed as they were on dying children, were of little significance, while, on the other hand, there were four very significant cases of ophthalmia as a result of auto-infection occurring among Fraenkel's own cases. Czéri also disagrees with the old view that scrofulosis and malnutrition are the conditions usually predisposing to vulvo-vaginitis. His cases were well-nourished children. (This fact is noted also by Fischer, Cassel, and Cahen-Brach.) Widmarck and Lennander in 1885, Israel in 1886, and Spaeth in 1889, all succeeded in demonstrating the presence of gonococci in vulvo-vaginitis in little girls, the report of Widmarck including bacteriologic examination of the pus of gonococcal conjunctivitis as well.

Other writers, less thorough in their methods, diagnosed cases

<sup>1</sup> Morris was led to take this at that time unusual stand by observing the extremely contagious character of vulvo-vaginitis in children. He says: "The intense, specific virulence of the gonorrheal poison is markedly shown by its spread through a whole family, from one child to another."

<sup>2</sup> In the thesis of Skiba-Zaborowska we find the statement that, beginning with the year 1879, a systematic search for the gonococcus was made in all cases of youthful vulvo-vaginitis occurring in the service of O. Wyss in Zürich Hospital. These observations seem, however, not to have been published before the date of the above thesis, 1898.

of infectious vaginitis as gonorrheal but without attempting to establish the fact by bacteriologic examination. (See the reports of Suchard, Comby, and Hofmokl.) There is little doubt in one's mind as one reads these articles that the disease was actually gonorrheal vaginitis, but the decisive proof is lacking. Hofmokl's attempt to differentiate between "spontaneous" vaginitis and blennorrhea on the ground of clinical symptoms, only leads to confusion and to the general impression that many, if not most, of the "spontaneous" cases were really gonorrheal. He adheres to the old theory that scrofula, malnutrition, anemia, uncleanness, etc., cause this form of vaginitis, yet he is obliged to admit that some of the cases in his clinic were strong, blooming girls, well cared for and cleanly. The disease often involved the urethra, rarely the bladder. It appeared in children of 1-8 years, while true blennorrhea was found in those between 8 and 12 years.

In 1891 the diagnosis of gonorrheal vulvo-vaginitis was facilitated by a careful article by Epstein in which he described three cases of gonorrheal vaginitis in the new born, and in which he differentiated this form from the desquamative catarrhal vaginitis of the new born. The latter condition he states to be of frequent occurrence, lasting usually two or three weeks. It is characterized by a tenacious discharge, which in rare cases may be creamy like pus, but which under the microscope seems to consist of squamous epithelium chiefly, the total absence of leucocytes serving to distinguish it from a gonorrheal discharge. In premature or weakly children this desquamative catarrh may last longer than usual. Cassel, in 1893, describes such a discharge which had persisted from birth in an 18 months-old child.

#### GONORRHEAL AND NON-GONORRHEAL VAGINITIS.

The general view at the present time is that a large number of cases of chronic purulent or catarrhal vulvo-vaginitis in little girls are of gonorrheal origin. There remains, however, a difference of opinion as to the diagnosis and the frequency of this disease. It is obviously of the utmost practical importance, this question as to whether the chronic vaginitis of little girls is to be regarded as almost invariably gonorrheal or only occasionally so, and as to the possibility of making a fairly positive diagnosis of such cases. The majority of hospital epidemics of which we have reports were started by infection

from an unrecognized case of gonorrheal discharge gaining entrance to the ward, and in all probability such an occurrence is often due to the widespread belief among physicians that a non-specific vulvo-vaginitis is very common in little girls and that the diagnosis of a gonorrheal vaginitis can be made only when the discharge presents a typical picture. It is undeniable that non-gonorrheal forms of vaginitis do occur in childhood. The question is how frequent they are. We have quite striking statistics as to the frequency of gonorrheal vaginitis reported by observers who have had the opportunity to study large numbers of such cases either in hospitals or dispensaries. Lennander found the gonococcus in all of his 18 cases of vaginitis; Rachford, in all of his 23. Czéri found it in 25 out of 26; Fischer, in 50 out of 54; Sheffield, in all 65; and Romnicéanu and Robin, in 130 out of 150. Others who have seen still larger numbers of cases, as Holt, Comby, Skutch, Welt-Kakels, do not give the exact figures but state that the great majority of those who came under their notice were gonorrheal. Holt says the non-specific cases number less than 5 per cent, and Pott, in his more recent articles, calls the chronic vulvo-vaginitis of children almost invariably gonorrheal, a statement concurred in by Prochownik, Buschke, Vogel, Berdal, Marfan, Jacobi, Rotch, Heimann, Edgar, Kimball, and LaFetra. On the other hand, we find traces of the older view, especially in French literature. An article by Vibert and Bordas in 1896 seems to have made a profound impression in that country. These authors took up the question from the medico-legal point of view and became convinced that not even the presence of the gonococcus could be taken as proof that the disease was "blennorrhagia." They based this statement on the fact that they found the gonococcus in the discharges of six cases of purely traumatic vulvo-vaginitis in little girls, and believed that they could rule out any possibility of gonorrheal infection in these cases. Cornil and Babes quote this view with approval, and Vassal indorses it emphatically. Marx maintains that not only is non-gonorrheal vaginitis far more common than the gonorrheal, but it is responsible for the severer complications, such as salpingo-ovaritis and peritonitis. Marx does not mention any bacteriologic studies of his cases. Henoeh in his edition of 1892 describes an infectious form of vulvo-vaginitis in children, but states that he attaches little importance to such

secretions for the reason that gonococci can be demonstrated in cases of vulvo-vaginitis in which no history of gonorrhea can be proven.

Even the most extreme upholders of the prevalence of gonorrheal infection in children recognize a non-specific vulvitis or vulvo-vaginitis caused by other microbic agents (diplococcus of Bockhart or of Fraenkel, streptococcus, staphylococcus), but claim that it differs from the gonorrheal clinically, as well as in the microscopic appearance of the discharge. It is more likely to be a pure vulvitis, it rarely involves the urethra (Spaeth, Fischer, Martin), is slightly if at all contagious, and is never complicated by ophthalmia. Various causes are assigned to this disease; trauma, including criminal assault; injury from foreign bodies; the irritation from pinworms, or from uncleanness, and the extension of non-gonorrheal inflammations of the labia. Fischer reports a case caused by the edema of heart disease, Cahen-Brach one which cleared up after the child was cured of obstinate constipation.

Such a discharge shows under the microscope few leucocytes and more numerous squamous cells than a gonorrheal discharge, and there are usually large numbers of bacteria of many kinds. In some cases, however, the differences are not so pronounced; there may be many leucocytes and some may contain diplococci, but these latter are Gram-positive and are therefore not to be confounded with the gonococcus. The diagnosis, then, in acute cases is not difficult. In long-standing cases, when the discharge has become thin and scanty, the matter is not so simple. To quote Levy and Klemperer, "although positive evidence of the presence of gonococci in microscopic preparation renders diagnosis of gonorrhea certain, negative evidence must be accepted with caution. It is known that when gonorrhea has existed for a considerable time gonococci are not always demonstrable in the rather mucoid secretion then present, but that nevertheless the gonorrhea may persist and still be infectious." Proof of the infectious character of a slight, non-purulent discharge in little girls is seen in Suchard's epidemic, in Hatfield's case, in Martin's experiment. Buschke says that many apparently bland discharges are due to an old gonorrhea, just as in the adult a chronic urethritis points to a gonorrheal history. Berdal makes the same statement.

That cultures may have to be made in some long-standing cases is shown by the experience of Heimann and of Welt-Kakels, both of whom succeeded in cultivating the gonococcus from a discharge, the smears from which showed no gonococci. Berggruen, also, and Park emphasize the necessity of making cultures in doubtful cases before a negative diagnosis is given. When this procedure is impossible it is generally conceded that a discharge rich in leucocytes and poor in bacteria should be regarded as very suspicious of gonorrhea.

Even in acute cases, the diagnosis may at first be difficult. Edgar states that the discharge is often free from gonococci during the first few days, the organisms appearing later. Nolen advises that in doubtful cases with thin scanty discharge a small amount of distilled water be injected into the vagina; which will result in the production, within 48 hours, of a profuse discharge in which gonococci can often be found.

Koplik, Berggruen, and Marx differ from the majority in respect to the frequency of non-gonorrheal chronic purulent vaginitis in childhood and as to its character. They recognize a non-gonorrheal form which is not due to trauma and which is clinically indistinguishable from the gonorrheal. Koplik describes it as a chronic purulent catarrh, often involving the urethra and sometimes the bladder, contagious, and caused by a specific, though as yet unrecognized, infection. The diagnosis depends upon the finding of many varieties of bacteria in the discharge, which, while full of pus cells, does not present the typical picture of gonorrhea. Berggruen does not recognize a case as gonorrheal unless he can cultivate the gonococcus from it. Marx thinks that the non-gonorrheal variety is much more common than the gonorrheal.

Heimann has gone into the question of specific and non-specific vaginitis in girls more thoroughly than anyone else in this country. He has succeeded in cultivating the gonococcus in cases where it did not appear in the smears. He found that in old discharges only degenerated cocci may be seen, and it may be impossible to find any typical organisms. Cultures were made from the vagina of 20 normal children and in no instance was the gonococcus found. Inoculation experiments in 2 males, with cocci isolated from these cases produced no urethritis, while similar experiments made with gonococci isolated



from the gonorrheal discharge of little girls produced typical gonorrhea. Heimann believes there is a specific microorganism of catarrhal colpitis, perhaps the diplococcus of Bockhart or of E. Fraenkel, but there is no difficulty in distinguishing it from the gonococcus if Gram's stain is used. Even when cocci are found inside pus cells, they will invariably be shown to be Gram-positive unless the case is one of gonorrheal infection.

Veillon and Hallé made a very thorough study of the bacteriology of the normal vagina in childhood and of the discharge in the vulvo-vaginitis of children. They conclude that the gonococcus is the habitual cause of chronic vulvo-vaginitis in children; that it is present alone or almost alone during the acute stage; that later on it is associated with other bacteria and may be present in very small numbers; that non-gonorrheal vaginitis is rare and is not caused by any specific microbe. The same microbes are found in the non-gonorrheal discharge as are found in the normal vagina and consist of *B. pseudodiphtheriae*, *Streptococcus*, *Staph. albus*, and rarely *B. coli*. These organisms are also found in a gonorrheal discharge of long standing.

#### PREVALENCE OF GONORRHEA IN CHILDHOOD.

The importance of adult gonorrhea as a widely prevalent disease, chronic, highly infectious, and capable of producing serious and even fatal lesions is now universally recognized, and of late years we have begun to see that what is true of the disease in adults is true of the disease in children also. The frequency of gonorrhea in little girls was insisted upon by Pott as early as 1883, but though there are fairly numerous articles in German literature and scattered ones in French we find little on this subject in our own country, until we come to recent publications. Holt, Koplik, Welt-Kakels, Sheffield, Kimball, Trenwith, Rachford, have shown by the study of cases in hospitals and dispensaries how widespread the disease is, at least in our large cities. That so few hospitals and institutions for the care of children acknowledge the presence of this disease is far from being proof that they do not have it constantly. Holt's description of the fight carried on against this plague in the Babies' Hospital in New York shows that even under the strictest precautions it does gain entrance and spread. Surely we may assume that institutions, which

make no attempt at all even to discover its presence, are in all probability more or less infected with it. Holt gives the results of examinations made by Dorothy Reed in institutions supposedly free from the disease and in which no precautions against infection were taken. She found in one place, among 100 infants and little girls, 12 with a purulent discharge containing gonococci, and 20 with a slight discharge in which the gonococcus could not be demonstrated. The statistics of the Babies' Hospital in New York show that there are about five or six cases of gonorrheal vaginitis among 125 applicants for admission.

The general impression among those who have studied this disease is that it is steadily increasing. This increase might be looked on as only apparent, for undoubtedly far more attention is paid to it now than formerly. Still it is undeniably true that in our large cities there is increasing opportunity all the time for infection from child to child. The tendency of modern city life is to bring large numbers of children together in close contact, in day nurseries, in hospitals (which are used far more than formerly), in kindergartens, in the schools, and in all manner of institutions for dependent children. An infected child who is kept at home will hardly spread the disease beyond her own family, but in a day nursery, or orphanage, or even a public school, she may be the cause of a widespread epidemic. And it is the growing conviction of physicians who have had experience in such institutions that, unless this disease can be excluded or controlled, a hospital or charitable institution for the care of little girls does more harm than good to the community. The public bath-house and swimming-pool are recent innovations in our country, and we have not yet heard from them as conveyors of gonorrheal infection, but in all probability we shall not escape the experience of other countries in this respect.

#### MODES OF INFECTION

We may dismiss without further consideration those cases in which infection follows attempted criminal assault by a gonorrheal male, and discuss only the indirect methods of infection.<sup>1</sup> In cases which occur outside of institutions the source of infection is most

<sup>1</sup> Infection from mother or other bedfellows may be direct, but is probably more often indirect.

often the mother, less often the father, sister, brother, or some other inmate of the house. Frequently the child has shared the bed of the infected adult, or has been bathed with the same sponge or towel. The mother's fingers also are often conveyors of the infection. Pott traced 90 per cent of his cases to the mother; Prochownik, 11 out of 16; Berggruen, 9 out of 13; Spaeth, 11 out of 14. Epstein's three cases were all infected during birth.

Two interesting epidemics are described by Suchard. In an establishment for sulphur baths in Lavey a newly cemented bathtub was used by 12 little girls with scrofula. One of the girls had a thin scanty vaginal discharge which suddenly became abundant and purulent. She was isolated and the bath thoroughly cleaned, but within three to six days all of the other girls developed the same trouble. A few months later 11 little girls, one boy, and a woman were bathed in another newly cemented tub. All of the girls developed purulent vaginitis during the following 17 days; the boy and the woman escaped infection. Suchard makes no mention of a bacteriological examination of the discharge.

The epidemic described by Skutch is the largest and most striking in the literature and is quoted in practically every article on vulvovaginitis of childhood. In 1891 there developed in Posen a widespread epidemic of this disease among girls, which was traced to the use of the public baths. Skutch reports 236 cases, developing within 14 days, and this hardly represents the whole number, for it was highly probable that many of the less serious cases never saw a physician. In this epidemic also, boys who were equally exposed to the infection escaped. This immunity of boys is noted by all observers, beginning with Fraenkel in 1885.

Infection in institutions is deplorably easy, and almost every object which enters into the equipment of a hospital or asylum has been accused as an agent of infection. The disease is practically invariably brought in by some child who is suffering from a vaginal discharge, more rarely ophthalmia, and in a few instances it is a boy with a gonorrheal proctitis. In institutions where children sleep two or more in a bed or are bathed in the same water, the mode of infection is not hard to trace. Sheffield's report of an epidemic in an orphanage is illustrative. They were accustomed in this institution to bathe from

20 to 30 girls at the same time in one great bath-tub. Sixty-five girls suffered in this epidemic. Even when the water is changed for each child, the bath-tub can undoubtedly be a conveyor of infection. Rectal thermometers were responsible for infections in Weill and Barjon's cases, in Baer's, in the epidemic at St. Annen Kinderspital in Vienna, mentioned by Fischer, and supposedly in other hospital epidemics. Bed linen, diapers, night clothes, water-closets, towels, all are regarded as probable causes of infection and those who have struggled to free an institution from this disease have in despair concluded that only complete isolation of the infected children, including isolation of their nurses, will serve to prevent further spread of the disease.

As one reads accounts of the attempts to combat the spread of gonorrhea in an institution for children, one is inclined to think that certain characteristics of the gonococcus have not been sufficiently considered. Experiments have shown conclusively that this organism is extremely sensitive to dryness and to a temperature above  $45^{\circ}$  C. (Neisser and Scholtz). Gonorrheal pus, which has been fully dried, contains no living germs, and even on bed linen, towels, etc., where drying takes place slowly, it can remain alive for only a few hours. The soaking of linen in disinfectants and then subjecting it to boiling temperature or to superheated steam, the strenuous cleansing of floor and walls, or the entire abandonment of an infected building would seem, therefore, to be unnecessary. On the other hand, the gonococcus can live for more than 24 hours<sup>1</sup> in warmth and moisture. All objects, therefore, which are damp and which come in contact with patient after patient in rapid succession should be regarded with the utmost suspicion. Such are the seats of water closets, bath-tubs, towels, sponges, clinical thermometers, and, most important of all, the fingers of nurses. This last danger seems to have been adequately emphasized only by Holt, who succeeded in tracing two cases of infection to the fingers of night nurses. In many hospitals, where the gonorrheal patients are supposedly isolated, special nurses are assigned to them by day only and the night nurse cares for non-gonorrheal cases as well. Undoubtedly infection has been carried in this way in more cases than those cited by Holt.

<sup>1</sup> Schaffer and Steinschneider.

## PREDISPOSING CAUSES.

The early view concerning the chronic vaginitis of children was that it arose spontaneously in ill-nourished, anemic, scrofulous little girls, and even down to the present day we find the statement frequently made that these conditions are strong predisposing causes of gonorrheal infection. It is not easy to decide on the value of the testimony on this point. Gonorrheal vaginitis is a disease which spreads in hospitals where naturally a large proportion of the children are scrofulous and anemic, and the same thing is unfortunately true of many institutions for children. On the other hand, children brought to dispensaries or seen in private practice with this disease are often apparently healthy and blooming. Hofmokl notes this fact with surprise; Fischer, Cahen-Brach, Cassel, and Czéri all say that the children seen by them were well nourished and not scrofulous.

As to the acute exanthematous diseases, there is certainly a general impression among physicians that a non-specific vulvo-vaginitis is a frequent sequela of scarlet fever and measles. Sheffield says that it is caused by saprophytic organisms. I can find no satisfactory description in the literature of the bacteriology of such a discharge nor any conclusive proof that it is not a gonorrheal vaginitis contracted within the hospital. Indeed, it seems very probable that many such cases are overlooked because of this idea that a discharge from the vagina is to be expected in measles and scarlet fever. Again, it is difficult to say whether these diseases increase the susceptibility to gonorrheal infection, or whether the facts cited in favor of this view can be explained on other grounds. Measles and scarlet fever are usually treated in hospitals; overcrowding is frequent during an epidemic and, as stated above, a vaginal discharge is not regarded as suspicious in such cases until other children have become infected, when its true character is discovered. It must be admitted that gonorrheal vaginitis spreads very quickly among children suffering from measles and scarlet fever. Fraenkel's 62 cases were almost all in the scarlet-fever ward. Cnopf, v. Dusch, and Weill and Barjon were convinced that both diseases increased the susceptibility to this infection. It is also noticeable that we do not find reports of epidemics of vulvo-vaginitis occurring in diphtheria wards.

## COMPLICATIONS.

Gonorrheal arthritis and conjunctivitis have been so thoroughly discussed in the literature that it seems unnecessary to take them up in detail. It may be pointed out in passing that, while conjunctivitis as an infection secondary to vulvo-vaginitis is growing rarer all the time, the same does not seem to be true of arthritis. This is undoubtedly due to the fact that infection of the eye is almost invariably from without and precautions against it may be taken, while infection of the synovial membranes takes place through the blood stream (Wertheim) and cannot be guarded against. Holt and Kimball report eight very mysterious cases of arthritis in children under 3 months, where no port of entry could be discovered, and suggest that the gonococci were carried by the nurses' fingers in the act of washing out the babies' mouths.

Urethritis is variously regarded; as the primary disease (Cahen-Brach, Koplik); or as an almost invariable accompaniment of the vaginitis (Spaeth, Buschke, Fischer, Callari, Berggruen, Veillon et Hallé); or as a comparatively rare complication in childhood (Kaufmann, Skiba-Zaborowska, Skutch, v. Dusch). Usually an involvement of the urethra is taken as strong proof of the gonorrheal character of the inflammation, but this is not admitted by Koplik, who finds urethritis in non-gonorrheal inflammations also.

Proctitis seems to be a rare complication. Buschke had 4 cases among 50, Sheffield, 2 among 65. Cystitis is also rare; stomatitis is mentioned, but apparently Rozinski's five cases are the only ones in which gonococci were cultivated from the lesions. Ahlfeld reported a case of stomatitis in an infant, smears from the patches showing typical gonococci. Adenitis is probably rather common, but does not usually attract sufficient attention to gain more than passing mention. Sheffield had 12 cases in 148.

The most important question in the consideration of gonorrheal vaginitis from the point of view of the little patient herself is the question as to the involvement of the upper genital tract. Is this disease in children simply a vulvo-vaginitis or is it also an endocervicitis, leading to endometritis and involvement of tubes and ovaries? We know that in some cases, localized or general peritonitis has resulted from a gonorrheal vaginitis. As Wertheim pointed out,

the gonococcus seems not to find the peritoneum a favorable ground and pure gonococcal infections are usually localized, chronic and not fatal. It is believed that this chronic peritonitis is not uncommon as a result of gonococcal infection in childhood and that it leads to the production of more or less serious lesions.

The mystifying cases of purulent salpingitis in young girls and of lesions pointing to an old peritonitis are explained as dating back to infantile gonorrhea by Saenger, Pott, Currier, Comby, Le Clerc, Edgar, and many more. Symptoms are often noted in the early stages of acute vaginitis which point to an involvement of the upper tract, such as rise of temperature, severe abdominal pains, enlargement and tenderness of the uterus, tenderness in the ovarian region, and, in other cases, all the symptoms of a severe peritonitis. A few of these cases have come to operation or autopsy and the nature of the infection has been proved by smears or cultures from the peritoneal cavity.<sup>1</sup> The infection is sometimes purely gonococcal, proving Wertheim's assertion that this organism alone can cause an ascending gonorrhea; in others the lesions in the peritoneum and especially in the organs are found to be due to mixed infection by the gonococcus and the streptococcus or to the latter alone. Rousseau believes that acute and fatal peritonitis is always due to a mixed infection.

Instances such as the above are, after all, exceptional, and it is much more important for us to know whether the uterus is involved in the ordinary case of gonorrheal vulvo-vaginitis when there are no clinical symptoms pointing to such involvement. If it is true that the apparently local disease may really cause such changes in uterus and tubes as to result in future sterility, then we shall be forced to regard a hospital epidemic of gonorrhea as an extremely serious thing. The obstinacy of the disease has led many to insist that it must penetrate to the uterus and thus remain unaffected by ordinary local treatment (Daphnis, Kaufmann, Jacobi). In all cases of acute vaginitis either gonorrheal or non-gonorrheal, which Koplik has

<sup>1</sup> I have been able to find in the literature only seven cases of gonococcal peritonitis or pyemia in little girls in which bacteriological examination is stated to have been made. These are the cases of Meyia, Dowd, Hunner and Harris (2), Hocheisen, Welt-Kakels, and Chiaisi and Isnardi. Others, in which there is strong probability that the infection was gonococcal, are the cases of Baginsky, Bracquahaye, Huber (2), Steven, Caillè, Saenger, Sigurd Lovén, Comby (8), Aguinot, Northrop. Marx says that pus was found in the Fallopian tubes in 5 out of 50 autopsies on little girls between 7 and 9 years of age, but no bacteriological examination was made, and Marx maintains that the vaginitis in these cases was not gonorrheal.

examined with the speculum, he has found the cervix red, swollen, and bathed in pus. He therefore thinks that the cervix is always involved in the process. Dind says that the cervical canal is the seat of predilection of the disease, not the vagina, but there is no satisfactory proof offered of this extreme statement. Lebedeff, working in Dind's clinic, stated that 29 of 56 cases examined through the speculum showed cervical involvement, but he based this belief on swelling, redness, and "fluor" bathing the cervix, and on the appearance of secretion on a tampon which had been laid against the external os. He mentions no microscopic examination of this secretion. Cahen-Brach and Skiba-Zaborowska had opportunity to examine the pelvic organs in four cases—two each—which came to autopsy from other causes, and they found no involvement above the vagina in any case. Gassmann took platinum loopfuls from the cervical canal in four cases and found three quite negative, the fourth contained pus cells but no gonococci. The largest number of such examinations is reported by Jung. He made smears from the cervical canal of little girls with gonorrheal vaginitis and found gonococci in 2 out of 20. One is inclined to think that this proportion is too small, both because no cultures were made and we know that gonococci sometimes are found in cultures when they are not discovered in the smears, and because Jung, for fear of error, rejected all cases in which gonococci were present in the vagina and portis vaginalis cervicis, accepting only those in which the organisms were exclusively in the cervical canal. However, even if his figures represent the exact truth, 10 per cent is no mean proportion.

#### DURATION OF THE DISEASE.

Authorities are generally agreed as to the obstinacy and long duration of this disease in childhood, even when the most approved treatment is given. Occasionally optimistic articles may be found in the literature describing modes of treatment which resulted in rapid cure (Sheffield, Rachford, Agramonte), but the majority seem to believe that an apparent cure is deceptive, that it means only a temporary remission, and that the disease may remain latent for long periods only to reappear months later. Buschke reports a case which lasted four years. Epstein thinks eight or nine years is not impossible.



Welt-Kakls saw cases in which the discharge had persisted for years before an acute exacerbation caused the parents to bring the child for treatment. The acute stage would then subside under treatment after four to six weeks, but a thin scanty flow would replace the purulent discharge and this would prove exceedingly persistent. She declares that no disease in childhood is more tenacious and refractory. The obstinacy of the disease is by most authors considered a diagnostic point in favor of gonococcal origin, but others, as Berggruen and Sheffield find their supposedly non-specific cases fully as obstinate.

#### DESCRIPTION OF EPIDEMIC IN SCARLET-FEVER WARDS.

During the months between July 20, 1907, and January 1, 1908, a series of epidemics of gonorrheal vulvo-vaginitis appeared in the scarlet-fever wards of Cook County Hospital. Scarlet fever was at the time very prevalent throughout the city and as there is but one hospital in Chicago for the acute contagious diseases, the scarlet-fever wards were very much overcrowded. When the first epidemic of vulvo-vaginitis broke out, the visiting staff advocated the isolation in separate rooms of all little girls entering the hospital, until it could positively be ascertained that they were free from gonorrheal infection, in order to avoid a second epidemic of this disease. The municipal authorities, however, insisted on the hospital admitting cases of scarlet fever up to the full capacity, and this precaution could never be carried out. As a result of the admission to the wards of children with latent gonorrheal vaginitis, five or six epidemics of the disease broke out and 82 infants and little girls were involved. For the opportunity to study these cases I am indebted to the courtesy of Dr. W. L. Baum, to whom I wish to express my sincere gratitude.

The hospital received during that time cases of diphtheria, measles, and chicken-pox, but the gonorrheal infection involved only the scarlet-fever wards. During the course of convalescence from scarlet fever and while still suffering from the vaginitis, certain of the little girls developed measles and a larger group developed chicken-pox. These children were then moved into the appropriate wards and occasionally a child without vaginitis was admitted to the measles and chicken-pox wards, but not one of the latter acquired vaginitis, although in the same room with these vaginitis cases. Girls over 13 years of age and

boys of all ages escaped infection even when every little girl in a given ward was attacked.

The children ranged from 13 months to 13 years of age, 61 (75 per cent) being under 7 years and only 5 over 10 years. Except for the scarlatinal infection, no predisposing cause was apparent. Many of the children were well nourished and free from any taint, and these children developed quite as serious a form of the disease as the anemic ones.

The diagnosis was made in all 82 cases by repeated examination of smears from the vaginal discharge; cocci identical with the gonococcus in morphology, distribution, and staining properties being found in each instance. In 11 cases cultures were made on ascites agar, 10 yielding the gonococcus, the 11th proving sterile. Only two of the 10 were pure cultures of the gonococcus. The others contained in order of frequency *B. pseudodiphtheriae*, *Staph. albus*, *Streptococcus* and *Staph. citreus*. Not one case of non-gonorrheal purulent vaginitis was found during this time.

Each epidemic was caused by the admission of a child suffering with gonorrheal vaginitis and a study of these occurrences would convince the most skeptical of the danger lurking in an apparently non-specific catarrhal discharge from the vagina of a little girl. For instance, in October a child entered a scarlet-fever ward which was quite free from gonorrhea. She had a slight vaginal discharge, smears from which were examined repeatedly by the internes, but no gonococci were discovered until the ninth day after admission. Meantime the child used the same bath-tub as the other children, went to the same water closet, and was attended by the same nurse. It would also often happen that she would be lifted into another child's bed while hers was being made, and vice versa. None of the children except the babies wore diapers. After the discovery of gonococci in her discharge she was isolated, but nine little girls from that ward developed gonorrheal vaginitis within five to seven days.

The history of another epidemic shows that three sisters were admitted, all with slight vaginal discharge. For 10 days examinations were made of these discharges but no gonococci found. The head nurse states that, as a precaution, these children were not tubbed nor allowed the use of the closet. Rectal thermometers were not at that

time used for any girl patients. The children were, however, in a large ward with other little girls, and cared for by the same nurses. After 10 days' time, gonococci appeared in the discharge of one of the three sisters and on the following two days in the discharges of the other two. Eighteen cases were more or less positively traced to this source of infection. Another child, who infected nine, was in the hospital for four weeks with a thin scanty, apparently non-gonococcal discharge. It became suddenly profuse and purulent. She was isolated, but the disease appeared in nine little girls who had shared the ward with her. Of the 82 cases, 16 entered with a pronounced or slight vaginal discharge, 66 acquired the disease in the hospital. Very strict precautions were taken after the disease developed; the children were isolated with special day and night nurses and apparently the disease did not spread from these wards. The responsible agent seemed in each instance to be a child with a latent, unrecognized gonorrhea, which apparently had been roused to activity by the scarlatinal infection. In several instances we had a demonstration of the fact that an acute exanthematous disease may light up into activity a previously latent gonorrhea. For instance, R. S., a little girl who had contracted vaginitis in the hospital, was convalescent from scarlet fever and so far recovered from her vaginitis that she had no visible discharge and one negative smear had been obtained. She contracted chicken-pox and the vaginal discharge reappeared with numerous gonococci. In other cases, not so near to complete recovery as this one, a decided exacerbation occurred on the outbreak of a second infectious disease.

As to complications, one can speak with certainty only of three cases of conjunctivitis, one very severe but resulting in complete recovery, the other two not quite so severe. All were proved by microscopic examination to be due to gonococcal infection. Four children had arthritis, but it was the opinion of the clinicians that these were scarlatinal, not gonococcal. One little girl had suppuration of a vulvo-vaginal gland, and although no microscopic examination was made of the pus, it is probable that this was an extension of her gonorrhea.

The disease varied greatly in intensity, light and severe cases appearing in the same epidemic. As to the duration, it is difficult

to say anything positive. Many children were removed by their parents before the vaginal discharge had subsided, others after the discharge had ceased but before the gonococci had disappeared. There are notes at hand of 29 cases which were dismissed apparently cured, i. e., they were free from discharge and the vaginal smears showed no gonococci. It was possible to follow 10 of these 29 children after they left the hospital. Three remained apparently free from gonococci for at least six weeks (the last time they were seen). The other seven all relapsed within a short time. Four of them are at the moment of writing still under treatment, the disease having already lasted from 10 to 13 weeks.

The important points in the history of this hospital epidemic are: the apparent predisposition to gonorrheal infection caused by scarlet fever; the fact that no girl over 13 years of age was infected; the total absence of Koplik's non-specific purulent vaginitis; and the fact that the infection was introduced into the hospital by children with vaginal discharge which was apparently non-gonorrheal. In conclusion, a table is appended of the epidemics of gonorrheal vulvo-vaginitis of children reported in the literature, and also of observations of scattered cases in dispensaries and private practice.

TABLE 21.  
SUMMARY OF CASES OF VULVO-VAGINITIS IN CHILDREN.

Name and Place*	Number of Vaginitis Cases Examined	Number of Gonorrheal Cases	Age	Bacteriological Examination	Supposed Mode of Infection	Complications
Atkinson, Institution Baltimore, 1878		6	5-12 years	None made	Infection carried by fingers of girls from eyes (conjunctivitis) and mouth (stomatitis)	Conjunctivitis and stomatitis were regarded as primary, 2 urethritis
Lennander, Hospital Stockholm, 1885	10	7	2-11 years	Gonococci found 5 times in vagina, 2 in urethra;	Indirect infection in hospital	4 conjunctivitis
Fraenkel, Hospital, Hamburg, 1885	18	18	5, 6, and 9 years	Gonococci found in all	No isolation or precautions against transmission	18 conjunctivitis
Leszynsky, Institution New York City, 1886		25	3-15 years. Majority under 5	None made	Fingers of children	
Czeri, Hospital, Budapest, 1885	26	35	3-10 years	By culture in 25 cases	Direct contact with one case; also indirectly by means of bed linen, lavatories, instruments, bandages	
Widmarck, Stockholm, 1885		12	20 months to 9 yrs.	Gonococci found in all	From mother or other members of family by sponges, towels, bed linen	Conjunctivitis
Israel, Dispensary, Copenhagen, 1886	23	8	2 mos. to 6 yrs.; 15 under 5 yrs.; 8 over 5 yrs.	Gonococci found in all 11 cases examined; gonococci in 9	Doubtful	
Pott, Hospital, Halle, 1883		44	27 from 1-5 yrs.; 4 over 10 yrs.	None made	From mother in 90 per cent of cases; others from relatives or house mates	Inguinal adenitis
Ditto 1888		42	26 from 1-5 yrs.; 3 over 10 yrs.	21 examined; gonococci in 17	Hospital cases by nurses' hands; outside cases by other members of family, indirectly	
Dusch, Hospital, Heidelberg, 1888		19	1½ to 12 yrs.	Gonococci found in all	Nurses' hands, sponges, etc.	
Olliver, Hospital, Paris, 1888		15		No mention	Use of same bath tub; one child with slight discharge	
Suchard, Sulphur Baths, Lavey, 1888		12		No mention	Use of same bath tub	
Spoeuth, Dispensary, Hamburg, 1889	21	14	3-11 yrs.	No mention Gonococci found in vagina and urethra in 14	Indirectly from mother in 11 cases; 2 cases of hospital infection	Urethritis in all
Skutch, Public Bathhouse, Posen, 1891		236	6-14 yrs.	Gonococci found in majority of cases	Use of public bath tubs; two or more bathed in same water	Many cases of urethritis and conjunctivitis

\* For references see alphabetical list at the end of this article.

TABLE 1.—Continued.

Name and Place*	Number of Vaginitis Cases Examined	Number of Gonorrheal Cases	Age	Bacteriological Examination	Supposed Mode of Infection	Complications
Cohen-Brach, Hospital, Graz and Frankfurt a. M., 1892	20	25	1½ to 12 yrs. 19 under 7 yrs.	Gonococci found in 25 cases 5 examined; gonococci in all	Attempted criminal assault in 6; others by sleeping with infected persons Direct in 1; indirect from mother in 1	23 urethritis; 1 conjuncti- vitis; 1 arthritis; 1 cystitis
Martin Dispensary, Philadelphia, 1892	9					2 urethritis; in several endo- metritis (clinically diag- nosed)
Comby, Hospital, Paris, 1891	151		69 between 13 mos. and 5 yrs. 1-13 yrs.; ma- jority under 8 yrs.	No mention	Indirect infection	1 conjunctivitis
Berggruen, Dispensary, Vienna, 1893	31	13	7 mos. to 11 yrs. Majority be- tween 2 and 3 yrs.	Gonococci in 13 cases, 7 purulent, 10 catarrhal	From mother, father, or other bedfellow	1 conjunctivitis; urethritis in many; pain and tender- ness in ovarian region
Cassel, Dispensary, Berlin, 1893	30		7 mos. to 11 yrs. Majority be- tween 2 and 3 yrs.	Gonococci found in 24 cases	From mother, father, or other bedfellow	2 conjunctivitis; 19 urethri- tis
Koplik, Dispensary, New York, 1893	24	17	2-10 yrs. Ma- jority under 6 yrs.	Gonococci found in 17 cases	Sleeping with infected persons	Urethritis and endocervicitis in majority
Vassal, Hospital, Bordeaux, 1894	14		75 per cent, 2 to 5 yrs.	None made	In 8 cases mother had leucor- rhea and slept with chil- dren	1 conjunctivitis; 1 otitis (?)
Weill et Barjon, Hospital Lyons, 1894	20			Gonococci found in all	Use of same rectal thermome- ter from infected child	
Fischer, Hospital, Altona, 1895	54	50	32 under 6 yrs.; 14 bet. 6 and 12 yrs.	Gonococci found in 50 cases	Direct and indirect contact in family and hospital	Urethritis in majority; 1 Bartolinitis
Veillon et Hallé, Hospital Paris, 1896	21	17	3 days to 11 yrs. Majority 2 and 6 yrs.	Gonococci found in 17; cul- tures in 17	No mention	1 conjunctivitis
Acrasmonie, Dispensary, New York, 1896	32		5 mos. to 10½ yrs.	Gonococci found in some cases	Direct and indirect	2 cystitis
Croft, Hospital, Nuremberg, 1898	10		3 mos. to 8 yrs.	Gonococci found in all	Nurse's hands conveyed infec- tion from cases of ophthalmia in babies	
Skilav-Zaborowska Hospi- tal, Zurich, 1898	71		2 weeks to 7 yrs. chiefly. Only 7 over 10 yrs.	34 examined; gonococcus in 29	Infection traced in 58, chiefly by thermometers, sponges, towels	5 conjunctivitis; 2 arthritis; 1 urethritis; 1 fatal peri- tonitis
Sheffield, Institution, New York City, 1900	65			Gonococci found in smears and cultures	Simultaneous use of one large bath-tub by 20-30 girls Common use of beds and small bath-tubs	6 conjunctivitis; 4 local peri- tonitis; 2 proctitis; 2 arthritis; 1 endometritis
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\* For references see alphabetical list at the end of this article.

TABLE 1—Continued.

Name and Place*	Number of Vaginitis Cases Examined	Number of Gonorrheal Cases	Age	Bacteriological Examination	Supposed Mode of Infection	Complications
Romiccau et Robin, Hospital, Bucharest, 1901	150	130	1-13 yrs.; about 50 per cent under 7 yrs.	Gonococci found in 130 cases	15 cases of attempted criminal assault; others by sleeping with infected persons	49 urethritis; 21 arthritis; 1 endocervicitis
Callari, Italy, 1901		54			27 attempted criminal assault; 27 indirect infection	49 urethritis; 3 proctitis; 1 conjunctivitis; 1 cystitis
Buschke Hospital and Dispensary, Berlin, 1902		50	2 mos. to 14 yrs.	Gonococci found in majority	7 attempted criminal assault; others by mother, father, or other inmates of home	1 Bartholinitis; 1 cystitis; 1 conjunctivitis; 1 arthritis; 1 peritonitis (clinically diagnosed)
Baer, Hospital, Chicago, 1904		19	6 mos. to 13 yrs.	Gonococci found in all; cultures from 2	By rectal thermometers, bed-linen, etc.	2 conjunctivitis; 1 cystitis; 1 peritonitis; 4 arthritis
Well Kakeks Hospital, New York, 1904	192	Great Majority	Up to 13 yrs. Majority 2 to 6 yrs. 2-9 yrs.	Gonococci found in majority; culture from 1	Indirect from cases in hospitals or from bedfellows at home	2 endocervicitis
Holt, Hospital, New York, 1905		273	All under 3 yrs.	Gonococci found in all Examination made after 1896; gonococci found	Nurse's hands, rectal thermometers, bath tubs, linen, diapers, fingers of children	6 conjunctivitis; 4 arthritis
Rachford Hospital, Cincinnati, 1906		23		Gonococci found in all	Rectal thermometers, bed linen, diapers, bath tubs	None
Trenwith, Dispensary, New York, 1906		12		Gonococci found in all	75 per cent infected by mother or father	None
Author's Epidemic, Hospital, Chicago, 1907		82	13 mos. to 13 yrs.; 75 per cent under 7 yrs.	Gonococci found in all; cultures in 10	Indirect from unrecognized cases of gonorrhea	3 conjunctivitis; 1 abscess of vulvo-vaginal gland

\* For references see alphabetical list at the end of this article.

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## INOCULATION TREATMENT OF GONORRHEAL VULVO-VAGINITIS IN CHILDREN.\*

ALICE HAMILTON AND JEAN MOTTRAM COOKE

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DURING the past two years several articles have appeared dealing with the opsonic index in the gonorrhea of adults and with the effect of therapeutic inoculation in this disease. The gonorrheal vulvo-vaginitis of little girls seemed to offer an interesting field for research of this kind and one as yet unexplored. Accordingly, during the summer of 1907, we undertook to examine children suffering from acute and chronic gonorrheal vulvo-vaginitis with reference to their opsonic index to the gonococcus; and later we took up the question of the influence exerted upon the index and upon the course of the disease by the injection of killed gonococci.

As a result of these investigations we feel justified in making the following statements:

1. The opsonic index to the gonococcus is usually below normal in the chronic gonorrheal vaginitis of little girls and in acute vaginitis during the first weeks.
2. The index usually rises toward the end in cases which are recovering and remains more or less persistently low in cases which do not improve or improve very slowly.
3. It is sometimes possible to raise the opsonic index to the gonococcus by injections of dead gonococci, and when this occurs an improvement in the clinical condition usually occurs also.
4. The acute cases inoculated with killed gonococci from strains which had been grown for several months on artificial media improved rather more rapidly than did control cases who received no injections of killed gonococci.
5. Better results are obtained by the use of strains which have been grown for a long period on artificial media than by the use of freshly isolated strains, and there appears to be no advantage in using the patient's own organism.

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6. While inoculation treatment does not produce a marked effect during the first weeks in acute cases, it seems to shorten the later stages; in chronic cases its effect is more evident than in acute.

7. The substitution of inoculation therapy for local treatment in little girls presents some decided advantages, especially in chronic cases.

8. It is desirable, though not absolutely essential, to control the inoculations by the patient's opsonic index.

Through the courtesy of Doctors Abt and Churchill we were able to follow nine cases of gonorrheal vulvo-vaginitis in little girls in the gonorrheal children's ward of Cook County Hospital. Two of these were acute cases and remained under observation 4 and 10 weeks respectively. Seven were chronic cases and were followed for periods ranging from 4 weeks to 6 months. The chronic cases will be reported separately. During the month of July a small epidemic of gonorrheal vaginitis broke out in a scarlet-fever ward of Cook County Contagious Hospital and this was followed during successive months by a series of more or less distinct epidemics. Doctors Baum and Cameron granted us the privilege of studying these cases and of trying the inoculation treatment on them. In this way we were able to secure 58 additional cases, making 60 acute cases and 7 chronic cases in all.

#### THE OPSONIC INDEX IN ACUTE AND CHRONIC GONORRHEAL VULVO-VAGINITIS.

The indices of the acute and chronic cases were estimated by comparison with a control composed of a mixture of three sera from healthy adults. In order still further to control the results, the same tests were made with the sera of 10 non-gonorrheal children who were under the same physical conditions as the gonorrheal and were at about the same level of general nutrition. These were 10 hospital children in the non-gonorrheal wards, who were not at the time suffering from any acute infection. Ten cases of chronic gonorrheal vaginitis were selected and, from the scarlet-fever wards, 10 acute cases who were in the first two weeks of the disease.

The gonococcus strain used for these tests had been grown for months on artificial media and readily emulsified into a smooth sus-

pension. The average indices of the 10 children in each of the three classes described above were as follows:

10 normal children, average.....	0.93
10 children with chronic gonorrheal vaginitis, average.....	0.62
10 children with acute gonorrheal vaginitis, average.....	0.44

We then made six examinations on successive days of the blood from five of the normal and five of the chronic cases in order to find what variations there might be in the indices.

In the normal cases the variations were as follows:

No. 1—0.8	to	1.18
" 2—0.8	"	1.15
" 3—0.85	"	1.15
" 4—0.8	"	1.15
" 5—0.8	"	1.2

This makes 0.8 the lowest, 1.2 the highest index, and 1.0 the average index.

In the chronic cases they were as follows:

No. 1—0.3	to	0.68
" 2—0.6	"	0.81
" 3—0.47	"	0.8
" 4—0.37	"	0.79
" 5—0.35	"	0.57

This makes 0.3 the lowest, 0.81 the highest, and 0.55 the average index.

#### THE INDEX OF CHILDREN INJECTED WITH KILLED GONOCOCCI.

Seven little girls with acute gonorrheal vaginitis were injected with a quantity of suspension of a 24-hour culture of the gonococcus in normal salt solution after heating it for 45 minutes at 60° C. The number of organisms was estimated by Wright's method. The strain used for injection was also used for testing the index. At the same time the indices were taken of four children, who were also suffering from acute gonorrhea but who were not injected with dead cocci. The dose at the outset was from five to ten million of cocci increasing to 20 or 25 million. Four injections were made in five of the children, five in one and three in one.

The average index of these seven children at the beginning of treatment was 0.58, but in each instance there was a fall after injections were begun and the average of the lowest indices was 0.37. A decided rise occurred usually only after the second or third injection;

a very marked rise was never seen, for the highest point reached in any of the seven cases was 1.9. The index followed fairly closely the clinical course of the disease. Chart 1 shows the curve of a child with very profuse discharge which cleared up rapidly, the child being dismissed on the 35th day with no discharge and no demonstrable gonococci in the vagina. Chart 2 shows the curve of a similar case

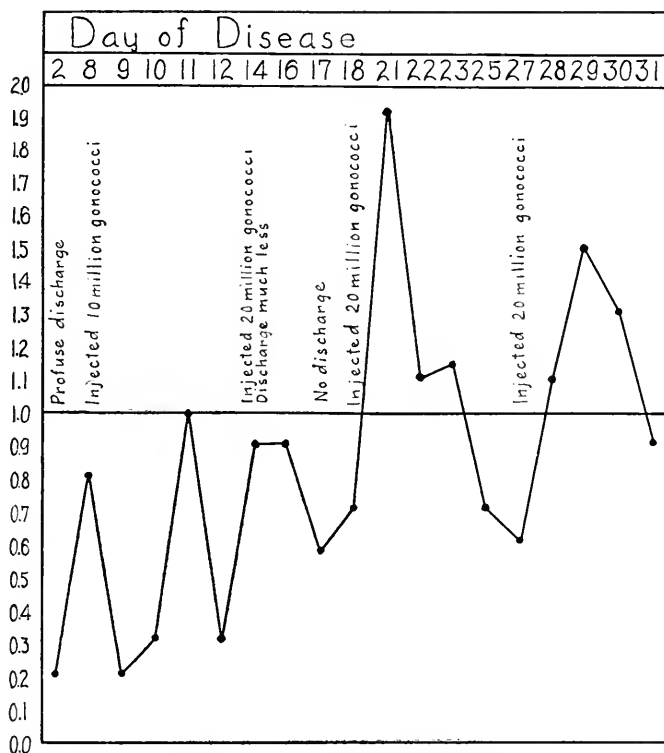


CHART 1.—Acute vaginitis; inoculation; recovery.

in which recovery was almost as rapid. Four of the seven children progressed fairly rapidly, and their indices corresponded to the course of the disease. These all had a very profuse discharge at the outset. One, a less severe case, lasted for six weeks with a slight, usually non-purulent, discharge, and her index was more often below than above normal and never rose higher than 1.4. The sixth, a severe and obstinate case, left the hospital after five weeks with a slight

discharge and a high index, but after two weeks at home she returned with a profuse discharge and a low index. She was given three injections after that and improved, but was still suffering from a purulent vaginitis when she was last seen. The seventh child (Chart 3) was completely refractory to the injections of dead gonococci, so

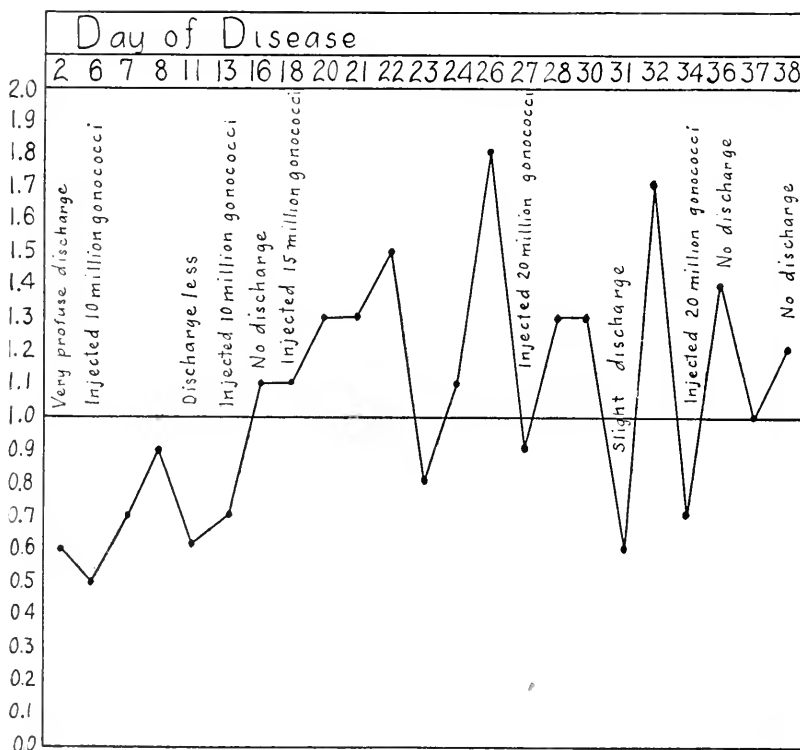


CHART 2.—Acute vaginitis; inoculation; recovery.

far as her clinical symptoms were concerned, and her index shows only one marked rise above normal.

The four children who were not given this treatment showed also a fairly close correspondence between clinical condition and opsonic index. Chart 4 is the curve of a very severe and obstinate case which left the hospital practically unimproved. Chart 5 is that of a severe case which improved decidedly but left the hospital before recovery. Chart 6 is of a mild case, dismissed without vaginal

discharge, although gonococci were still present. The fourth case was one of moderate severity, but obstinate, and the curve showed no rise higher than 1.3.

To sum up the results in these eleven cases, we find the lowest index in both injected and non-injected to average 0.37; the average

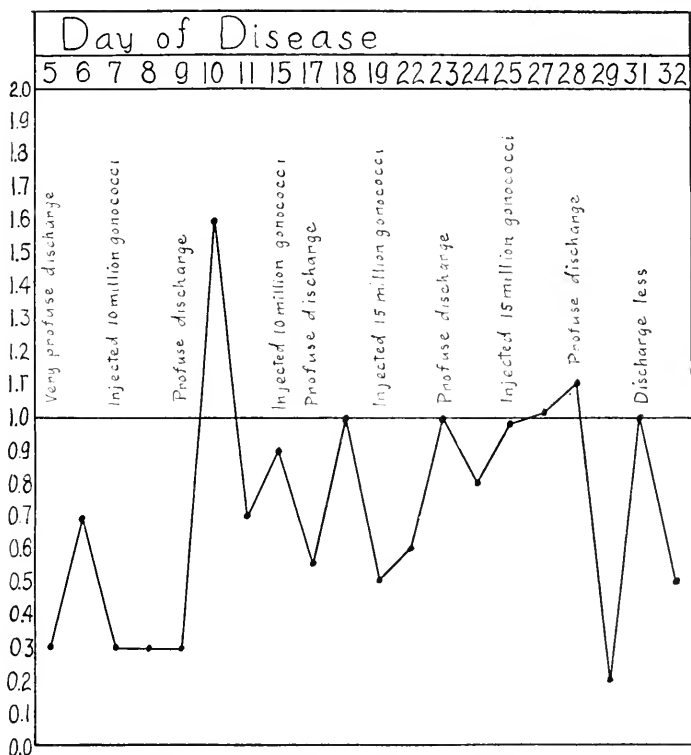


CHART 3.—Acute vaginitis; inoculation; no improvement.

of the highest indices in the seven injected cases to be 1.7, in the four control cases 1.3.

#### EFFECT OF INOCULATION THERAPY ON THE COURSE OF ACUTE VULVO-VAGINITIS.

The difficulty in estimating the effect of any given treatment in the case of spontaneously curable diseases lies in the impossibility of absolutely controlling the results by comparison with cases not subjected to the treatment. No two cases are alike, and gross inac-

curacies may be overcome only by the comparison of large numbers of cases as nearly alike as possible. We divided our 60 acute cases into two groups, pairing the children as nearly as possible according to age and to the time of outbreak of the disease. One group was given injections, the other the usual local treatment which consisted

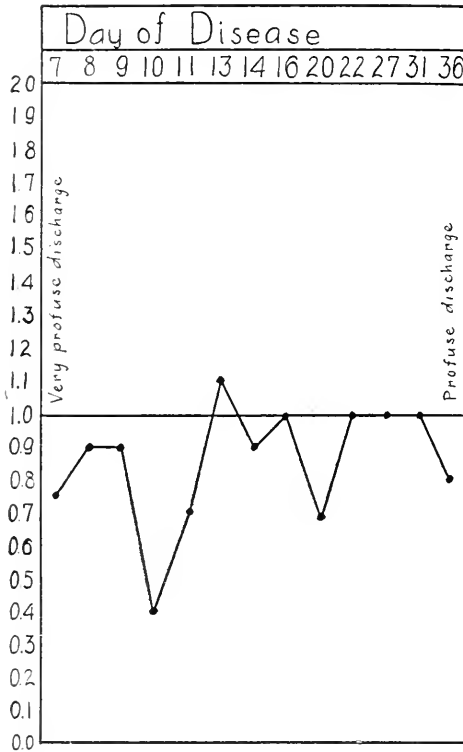


CHART 4.—Acute vaginitis; no inoculation; no improvement.

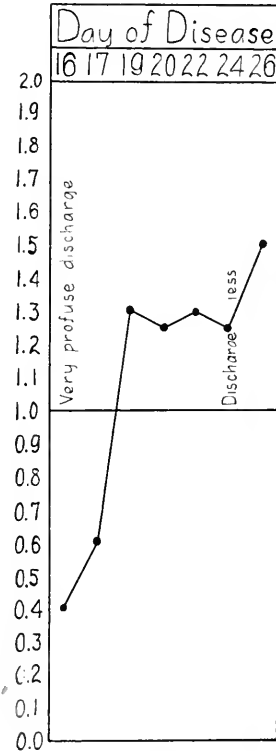


CHART 5.—Acute vaginitis; no inoculation; improvement.

of douches twice daily with potassium permanganate solution 1-4000, followed by the instillation of 20 per cent argyrol. The first five children who were given injections of killed gonococci were given local treatment also, but we were emboldened by our apparent success in these cases to ask permission for the suspension of all local treatment in the next 20, who accordingly received injections of killed gonococci only. Finally the last five were given both local treatment and inoculations. The ages of the 60 children ranged from 13



months to 11 years. Over three-fourths were under seven years and only two were over 10 years. The discharge was described as very profuse at the outset in 41 cases, moderately profuse in 12, and as a slight purulent discharge in 7 cases. As complications were noted, three cases of gonococcal conjunctivitis and one abscess of a vulvo-vaginal gland. Four cases of arthritis developed but were diagnosed as probably scarlatinal, not gonococcal. In none was there any evidence of pus in the joint. The children were for the most part healthy and robust.

The 30 cases selected for specific treatment were with three exceptions in the first week of the disease and 23 had had the discharge for not more than three days when they received their first injection. Two were in the first week of an acute attack, but it was suspected that these were really chronic cases with a temporary exacerbation of the discharge; the third exception was a girl with typhoid fever who did not receive an injection till her temperature had become normal, when the discharge had already persisted for 24 days.

Material for injection was prepared from seven strains of gonococci, five of which were freshly isolated strains growing on ascites agar, and two were old strains isolated months ago and grown on Löffler's blood serum, or horse-serum agar. The dosage was necessarily largely a matter of guesswork, and we have used amounts varying from 2,500,000 to 50,000,000 without being able to decide what is the proper dose. We used larger doses of the old strains than of the new, believing that the former had in all probability lost part of their virulence. As

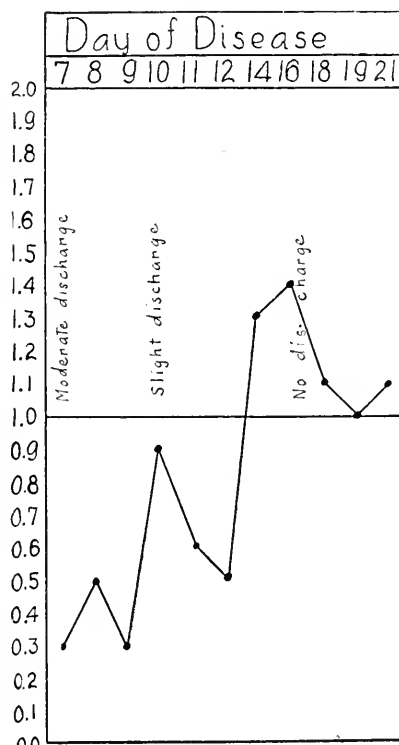


CHART. 6—Acute vaginitis; no inoculation; improvement.

a rule, in a child of three to seven years, we began with a dose of 5,000,000 and increased until at the fourth or fifth dose she was receiving 20,000,000. In one little girl improvement seemed to follow a return to a smaller dose after 15,000,000 had been reached. In others also we gained the impression that the dose used—from 25 to 40 million—was too large. On the other hand, a dose of 35 to 45 million, toward the end of the child's stay in the hospital seemed in several instances to hasten the clearing-up of the discharge.

In comparing the results of the inoculations and the local treatment without inoculation, the only possible way seems to be to take the duration of the acute stage of the disease, that is, the number of days of purulent discharge, and the condition of the child on leaving the hospital. The actual duration of the disease could not be ascertained in a number of cases for the children left the hospital at the end of their convalescence from scarlet fever, measles, or chicken-pox, and while still suffering from the vaginitis.

A comparison of the duration of the purulent discharge in the two classes of children shows practically no difference. For the injected children it averaged 20 days and for the non-injected 21 days. However, a closer analysis of the histories of these children reveals the fact that only 18 of the non-injected children began with a severe discharge, while 23 of those injected were severe from the outset. The averages for these two sets of children would be, 21 days for the injected and 27 for the non-injected, showing a very slight advantage for the inoculation treatment. Taking the condition at dismissal, it appears that, although the injections of gonococci do not shorten the acute stage to any decided extent, they do result in a more rapid disappearance of the later, thin discharge and also in a more rapid disappearance (temporary, doubtless in many cases) of the gonococci. Thus we find 18 of the 30 inoculated children leaving the hospital free from discharge and from gonococci, while only 8 of the control children did so; 13 of the 23 severe cases among the inoculated and only 2 of the 18 severe cases among the control children left the hospital apparently free from gonorrheal discharge.

At the beginning of our work we used for injection one strain of gonococcus only, a strain which had been grown for months on artificial media. Later we used a second long-isolated strain and five which

had been freshly isolated and grown on ascites agar. The experiment with these last five strains proved unfortunate, both when they were used alone and when they were mixed with one of the old strains, and this was true also of the cases in which the patient's own organism was injected.

Fifteen children were injected with old strains, fifteen with new, or with new and old mixed, four of these receiving their own strains. The following statement shows the average duration of purulent discharge in these cases:

	AVERAGE DURATION
Homologous strains, 4 children, 3 severe cases.....	23 days
Freshly isolated strains, 3 children, 5 severe cases.....	24 days
Mixed old and fresh strains, 6 children, 4 very severe cases	27 days
Old strains, 15 children, 12 severe cases.....	14 days

If we take only 12 seven cases treated with old strains and compare the duration of the acute stage in them with the duration in the 18 similar cases who received local treatment only, we shall have an average of  $14\frac{3}{4}$  days for the former as against  $27\frac{3}{4}$  days for the latter. This shows that our results would have been more encouraging had we not experimented with fresh strains.

A comparison between the children who received both douches and inoculation and those who received the last alone shows that there is nothing to be gained by adding the local treatment. Those receiving both forms of treatment averaged 20 days; those receiving inoculations alone, 21 days.

As to the condition of the children after they left the hospital it is impossible to speak with any accuracy, because so small a proportion remained under observation. Seven children who had been inoculated were followed for six weeks or two months after they left the hospital. Three only remained free from any sign of the disease, two suffered slight, and two a severe, relapse within a week after they left the hospital.

#### INOCULATION THERAPY IN CHRONIC VULVO-VAGINITIS

In reporting the results obtained with the seven chronic cases we are obliged to adopt another method. These cases could not be controlled by comparison with similar ones who were receiving no bacterial treatment, for it was not possible to find control cases that

corresponded closely enough in the duration or character of the disease. Had we been able to command a large number of chronic cases, as we did of acute cases, our results could have been estimated in the same way, by averages, but the period of time required in the chronic cases was so much longer than in the acute that many of the children left our hands too soon for us to be able to draw conclusions from them. We can compare the condition of our seven children before we began treatment with their condition after treatment.

*Case 1.*—A girl of seven years had had a more or less profuse purulent discharge from the vagina for seven weeks and had been treated during that time with douches of permanganate solution followed by instillation of 20 per cent argyrol. She was given injections of old strains of gonococcus, beginning with 15,000,000 and going up to 25,000,000 cocci. The discharge improved and she was allowed to return home, coming to the hospital for her injections. At the end of  $3\frac{1}{2}$  weeks no gonococci were found in the smears from the vagina. She then passed out of our care before we could feel any certainty as to her real recovery.

*Case 2.*—A girl of 10 years had been in the hospital for eight months and had received douches twice daily during that time. She had a scanty mucoid discharge in which gonococci were always demonstrable, and her condition remained absolutely unchanged under local treatment. She was given injections of the same cocci as Case 1, beginning with 20,000,000 and reaching 30,000,000 at the fifth injection. After this injection there is a note to the effect that the child had no visible discharge and that gonococci were found only in two out of six smears. The 7th to 11th injections were made with suspensions from a fresh strain, but after that the number of gonococci in the smears increased somewhat and we returned to the old strains. The girl is now, at the end of five and a half months' treatment, in almost the same condition as at the outset; that is, she has still at times a slight discharge and it has never yet been possible to obtain two successive smears free from gonococci.

*Case 3.*—A girl of seven years had had a profuse purulent discharge for two months at the time her injections were begun, and had been given douches in the hospital for four weeks. The dose (old strain) given her was 10,000,000. She improved slightly after the first and

decidedly after the second injection. On the 20th day of treatment she had no visible discharge, but there were still a few gonococci in the smears. Unfortunately she was then removed by her family, after four weeks of treatment consisting of four doses of 10,000,000 each. Her index was followed during the time of her treatment. It was 0.6 at the outset, fell later to 0.2, and finally rose to 1.65.

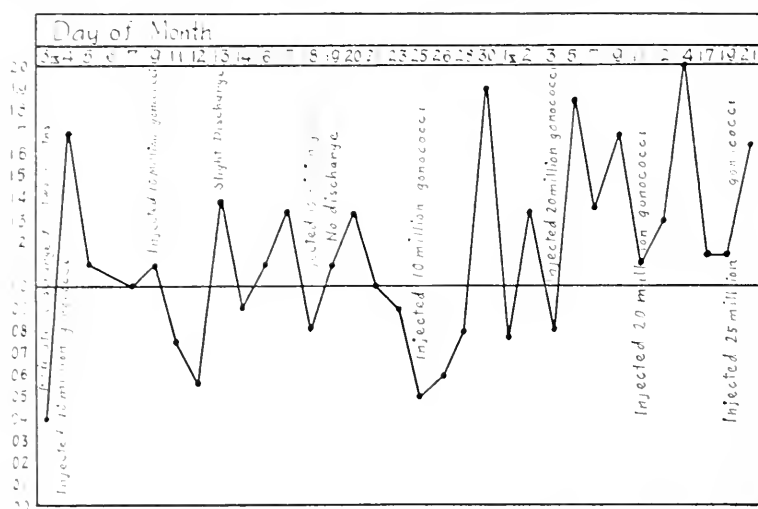
*Case 4.*—A child of 3 years had had an intermittent profuse purulent discharge for seven months. Her index was taken and found normal—0.8—but it fell to 0.35 after the first injection of 15,000,000 (old strain) and then rose to 1.5. She began to improve after the second injection, but was not free from discharge till after the third, and was not free from gonococci after the fifth, when she was, unfortunately, allowed to leave the hospital. She was brought back three weeks later, suffering from lobar pneumonia and with a profuse vaginitis. Again her index was normal, again it fell to 0.65 after an injection, and then rose to 1.7. She remained in the hospital long enough to receive five more injections and left after three successive smears from the vagina had failed to show gonococci.

*Case 5.*—A girl of five years had contracted gonorrhea in the scarlet-fever ward of the Contagious Hospital five months previously and had since then a discharge always fairly and often very profuse. She had received local treatment for three months before injections of killed cocci were begun. Her discharge diminished after the second injection and never reappeared in purulent form. After the fourth injection she had no recurrence at all, but she had constantly an appreciable number of gonococci in the vaginal smears. At the present time she is still in the hospital, after  $3\frac{1}{2}$  months of treatment, without vaginal discharge and at times without demonstrable gonococci, but it is impossible to dismiss her as yet. She has received 14 injections, of old, of fresh, and of old and fresh strains mixed, the dose rising from 7,500,000 to 50,000,000. Her index was taken for seven weeks and remained for the most part below normal or very slightly above. It never rose higher than 1.5.

*Case 6.*—An older sister of Case 5 had contracted gonorrhea from her. At the time her treatment was begun she had had a moderately profuse purulent discharge for the greater part of the preceding three months, during which time she had been receiving local treatment.

Her index was 0.4 before the first injection and rose to 1.7 after it (Chart 7). Improvement in her condition began after the third injection, and she has had no vaginal discharge from that time on. The gonococci did not, however, disappear completely till after her 12th injection. She is now in an orphanage under strict medical supervision, so that it is possible to assert that she has had no recurrence of the disease.

*Case 7.*—A little girl of four years had been in the gonorrheal ward for two months but had proved entirely refractory to local treatment. It was not known how long the vaginitis had existed



• CHART 7.—Chronic vaginitis; inoculation; recovery.

before she entered the hospital as she was brought there for ophthalmia and her mother professed to know nothing of the other trouble. Her discharge, which was very profuse, diminished after the third injection and ceased after the fourth. The gonococci disappeared after the seventh and were not found in repeated examinations during the following two weeks. Her index followed very closely the clinical course of the disease (Chart 8).

We have, then, seven chronic cases in which local treatment had been given a fair trial, sometimes a very extended trial, and had failed to bring about improvement. One of our seven (Case 2) proved refractory to inoculations also, and she evidently represents

that very obstinate form of gonorrhea in childhood which is most dangerous because so seldom recognized. The other six cases improved decidedly under the injections of killed gonococci as is shown by the fact that while the average duration of vaginal discharge prior to treatment (in the five cases in which this could be ascertained) was a little less than four months, the average duration after treatment was not quite three weeks. It is not claimed that a permanent cure was effected in any of these children. The experience of all who have carefully studied this disease is that recurrences are to be expected after apparent recovery. It is only claimed that the disease can at

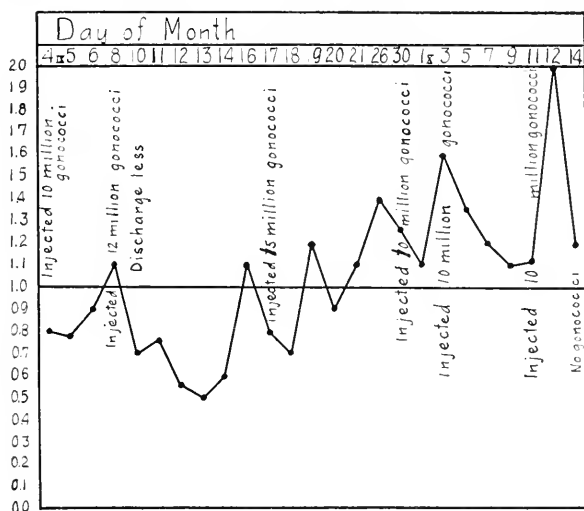


CHART 8.—Chronic vaginitis; inoculation; recovery.

least be temporarily controlled by raising the opsonic index to the gonococcus through the injection of dead gonococci.

Even if the progress under inoculations were no more rapid than that caused by the usual methods of local treatment, there would still be decided advantages in its favor. Everyone recognizes the drawbacks to local treatment in babies and little girls; a treatment almost impossible in any place but a hospital and not free from risk even there. Bumm stated that he had seen all the symptoms of an acute peritonitis follow the administration of a vaginal douche in a little girl, and while such an event can hardly be regarded as anything but most exceptional, it must be admitted that there is some risk of

driving the infection up into the cavity of the uterus. Aside from this, there is the less serious risk of producing slight mechanical injuries and of inducing the habit of masturbation, both of which would result in prolonging the course of the disease. In our cases douching was suspended soon after the inoculations were begun, and it was evident that not only was the progress of the case not retarded thereby but in some instances it was hastened. Three little girls had been incorrigible masturbators as long as they were given vaginal douches, but when the latter were abandoned they seemed to have no further temptation to this habit—a change which certainly contributed to their recovery.

In seven of the acute cases and in five of the chronic cases the opsonic index was followed, and the injections of dead gonococci were given according to the index. The interval between injections was at first from five to seven days, then as we noticed that a second rise in the index sometimes occurred (see Chart 3) if the injections did not follow each other too rapidly, we lengthened the interval to eight or nine days. Those children whose indices were not studied were injected regularly according to this rule.

In our experience it is advantageous to follow the index while giving inoculations, although it is not essential. The index does serve as a valuable guide in determining the amount and the time of injections (see Chart 9 for improvement after diminished dose). However, when this is not possible the treatment can still be carried out with success.



## ON THE LEUCOCYTOTOXINS OF NORMAL SERUM.\*

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It has long been known that normal sera may contain elements toxic to cells of other animals, as exemplified by the normal hemolysin or the less familiar haptin producing necroses (Uhlenhuth,<sup>1</sup> Pfeiffer<sup>2</sup>). The occurrence in sera of bodies possessing a toxic action on foreign leucocytes has been observed, but very little is known of their nature. Noguchi<sup>3</sup> found that the leucocytes of certain species of crabs are injured by the serum of a number of poikilothermous animals. Christian,<sup>4</sup> studying a considerable number of combinations, detected leucotoxic action in only one case. He placed the leucocytes in a warm chamber under the microscope and added the serum to be tested. Loss of ameboid movements was taken to indicate the toxic action. Then there are the rather conflicting observations of Laschtschenko<sup>5</sup> and of Metchnikoff,<sup>6</sup> those of the former to the effect that the injurious effect of foreign sera upon leucocytes is prevented by heating to 60°, and those of the latter indicating that sera heated to 60° still retain the power of agglutinating leucocytes. Also Ruediger and Davis<sup>7</sup> state that human leucocytes are injured by the sera of many species of poikilotherms and that this action is prevented by previously heating the serum to 55° C.

In place of the method of Christian, the leucocytes in my experiments were examined for signs of degeneration by means of stained films. Leucocytes were obtained either from the exudate caused by intrapleural injections of aleuronat or from blood cream, washed in NaCl solution, and usually 0.2 c.c. of fairly dense suspensions (unless otherwise stated) were mixed with varying quantities of the serum tested, the whole being made up to 0.5 c.c. with normal NaCl solution and incubated at 37° C. for about one hour. Smears were then made and stained with thionin.

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<sup>1</sup> *Ztschr. f. Hyg.*, 1897, 26, p. 384.

<sup>2</sup> *Ibid.*, 1905, 51, p. 183.

<sup>3</sup> *U. of Penn. Med. Bull.*, 1902, 15, p. 295.

<sup>4</sup> *Deut. Archiv. f. klin. Med.*, 1904, 80, p. 333.

<sup>5</sup> *Arch. f. Hyg.*, 1900, 37, p. 290.

<sup>6</sup> *L'Immunité*, Paris, 1901, p. 100.

<sup>7</sup> *Jour. Infect. Dis.*, 1907, 4, p. 333.

In contrast to the leucocidin of *Staph. pyogenes aureus* (Van de Velde<sup>1</sup>), whose action is limited to the cytoplasm of the white corpuscles, the action of normal serum leucotoxin is principally upon the nucleus, so that it appears to be more closely allied to the leucocidin produced by *B. pyocyaneus* (Ghéorghiewsky<sup>2</sup>) and those formed by *B. anthracis symptomatici* and *B. edematis maligni* (Eisenberg<sup>3</sup>). Corresponding to the amount of serum employed and to the length of the incubation period the action of this body manifests itself in varying grades o

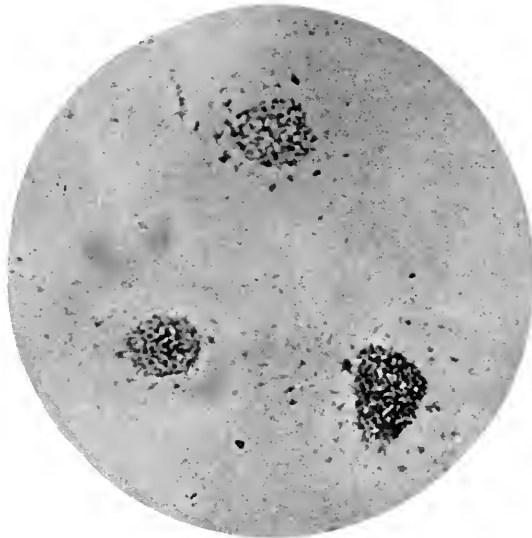


FIG. 1.—Nuclei disintegrated.

intensity ranging from almost complete destruction as shown by transformation of the leucocyte into a clump of granular débris, which stains a faint reddish brown with thionin, to a condition in which the nuclei merely lose their normal contour and become vacuolated and diffused over the entire cell; this change is accompanied by an alteration in the staining reaction, the nuclear material having a reddish tinge in place of the normal deep blue (see Figs. 1, 2, and 3). The change in the staining reaction may be obscured, however, by prolonged application of the thionin, which now may give the normal blue

<sup>1</sup> *Ann. de l'Inst. Past.*, 1896, 10, p. 580.

<sup>2</sup> *Ibid.*, 1899, 13, p. 298.

<sup>3</sup> *Compt. rend. Soc. Biol.*, 1907, 62, p. 491.

color. That both these types of degeneration are but different manifestations of the same phenomenon and are due merely to variations in the completeness of the reaction is shown by the presence of every conceivable intermediate grade between the two extremes in different smears. In all cases, however, the leucocytes in a single smear are in the same condition.

When the action is very marked all types of leucocytes are equally affected, but when it is less strong the cells show a variable suscepti-

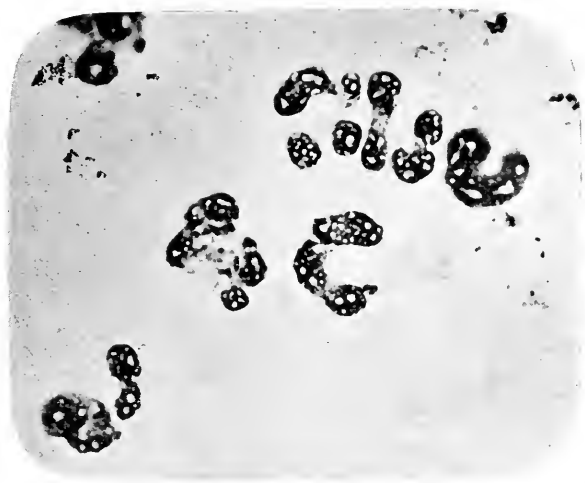


FIG. 2.—Vacuolization of nuclei.

bility to the poison, that of the large mononuclear leucocytes being greatest, that of the polymorphonuclears least, and that of the lymphocytes intermediate. A smear may thus show complete destruction of the large mononuclear leucocytes, distinct degeneration of the lymphocytes, and perfectly normal polymorphonuclear cells. This difference in the susceptibility of the various types of leucocytes is of some interest in view of the present conception of the adaptation of the large mononuclear leucocytes as devoted to immunity against animal cells especially and of the polymorphonuclears against vegetable cells (bacteria). In the experiments recorded subsequently + indicates mere degeneration of the large mononuclear elements with no visible change in the other types, ++ destruction of the large mononuclears

with degeneration of the lymphocytes, and + + + destruction of all the leucocytes.

The first step was to determine how widely normal serum leucotoxin is distributed. Christian, using loss of ameboid movements as the criterion, found but a single instance in which leucotoxin was present, namely in chicken serum for dog leucocytes. It is true that Noguchi observed leucotoxins in the blood of several poikilothermata for the white cells of crabs, and Ruediger and Davis for human

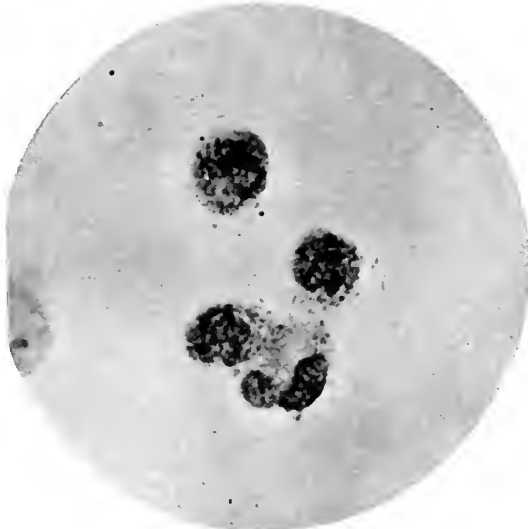


FIG. 3.—Nuclei degenerated and diffuse—sharp outlines lost.

leucocytes, but no further details are given by these authors. In order to determine whether the results of Christian's experiments would dovetail with those obtained by means of the staining reaction, four of the combinations employed by that investigator—chicken serum with human, dog, and rabbit leucocytes, and dog serum with rabbit leucocytes—were repeated. In each case the results tallied with those Christian obtained by the motility test. A number of additional combinations were then studied, and the results of which are recorded in Table 1. This shows that while normal leucotoxins are not widely distributed, they nevertheless are far from being as rare or unusual as might perhaps be surmised from the earlier observations, and consequently a knowledge of the nature of these

bodies is of importance. Unless otherwise designated the following experiments were performed with human serum and dog leucocytes.

TABLE 1.  
DISTRIBUTION OF NORMAL LEUCOCYTOTOXINS.

SERA	LEUCOCYTES				
	Human	Dog	Rabbit	Guinea-pig	Swine
Human.....		+	-	-	+
Dog.....	-	-	-	+	
Rabbit.....	-	- (?)			
Guinea-pig.....	-	-			
Swine.....	-	-	-	-	
Goat.....	-	+	+		
Limulus.....	-	+			
Chicken.....	-	+	-	+	
Sheep.....	+	-	+	+	
Horse.....	-	+			
Beef.....	-	+			
Rat.....	-	+			

Leucotoxin is bound by the leucocytes, since on mixing an equal quantity of serum with a thick suspension of washed leucocytes and incubating at 37° C. for one hour, the supernatant fluid obtained by centrifugation is devoid of any toxic action on a fresh suspension of leucocytes. It is therefore possible to remove the leucocidal body from serum by saturation with leucocytes.

As noted, Laschtschenko and Metchnikoff have published conflicting statements concerning the thermolability of leucotoxin. I have found that it is invariably destroyed by exposure to 55° C. for 30 minutes.

By dilution of leucotoxic serum a quantity of serum may be obtained so small that it is incapable of producing toxic effects on a leucocytic suspension. If, however, this quantity of normal serum, too small to kill leucocytes by itself, be added to a larger quantity of serum, previously heated to 55° C. for 30 minutes, and which also is innocuous by itself, the mixture readily destroys white corpuscles exposed to its action (Table 2). Hence, the leucocidal action of normal serum is produced by the interaction of two independent bodies, one thermolabile and capable of acting in very minute quantities, the other thermostabile and less abundant or less active than the thermolabile element.

A question of the interest in regard to the nature of normal leucotoxin is that of its identity with normal hemolysin. The susceptibility

of both to separation into a complemental and an amboceptor-like fraction would seem to speak in favor of their identity. It appears

TABLE 2.  
REACTIVATION OF HEATED LEUCOCYTOTOXIC SERUM.

Leucocytic Suspension	Normal Serum	Heated Serum	Result
0.2 c.c.	0.2 c.c.		+++
0.2	0.1		+++
0.2	0.05		+++
0.2	0.025		++
0.2	0.0125		++
0.2	0.006		+
0.2	0.003		—
0.2	0.0015		—
0.2	0.0007		—
0.2	0.00035		—
0.2		0.2 c.c.	—
0.2		0.1	—
0.2	0.003	0.1	+++
0.2	0.0015	0.1	+++
0.2	0.0007	0.1	++
0.2	0.00035	0.1	++
0.2			—

justifiable, however, to assume that the two bodies are quite distinct and independent of each other in view of the following facts. In the first place, the distribution of leucotoxins as shown above is not co-extensive with that of the normal serum hemolysins. This fact by itself might be considered ample proof that the two are distinct bodies. It is not entirely conclusive, however, for it is conceivable that the leucotoxic and hemolytic actions are two functions of the same substance, but that in some cases the former does not manifest itself because of some species peculiarity in the binding group of the leucocytes. In the second place, there is a quantitative difference in the minimum leucotoxic and hemolytic doses of serum for the blood elements of the same animal for it was found that the former manifests itself in far higher dilutions than the latter. This is shown in Table 3. The non-identity of the two substances is apparently indicated by this

TABLE 3.  
QUANTITATIVE RELATIONSHIP BETWEEN NORMAL LEUCOCYTOTOXIN AND HEMOLYSIS.

Serum	Hemolysis (1 c.c. 5 per cent Susp.)	Leucolysis (0.2 c.c. Susp.)
0.2 c.c.	+	+++
0.1	+	+++
0.05	+	+++
0.025	+	+++
0.0125	—	++
0.006	—	++
0.003	—	+
0.0015	—	—

observation, but here again it must be admitted that the experiment is not conclusive and the result must be regarded as merely presumptive evidence. While the erythrocytes were used in 5 per cent suspensions of which 1 c.c. was taken and may reasonably be assumed to represent a fairly constant quantity of cells, this is not true of the leucocytic suspensions, for there is no way of obtaining a suspension of leucocytes equal in cellular richness to that of the erythrocytes, and there is also daily variation in the leucocytic concentration of the aleuronat exudate from the same animal. But even if leucocytic and erythrocytic suspensions of equal concentration were employed it is not impossible that a given quantity of one and the same amboceptor might be capable of sensitizing many times as many white as red cells.

Far more conclusive results are obtained by absorption experiments. By saturating a quantity of heated serum with an excess of red blood corpuscles the presence of an unbound specific leucotoxic amboceptor may be demonstrated by exposing white corpuscles to the residual fluid and adding a quantity of normal serum too small in itself to produce leucocytic karyolysis, and hence containing complement but no amboceptor, as proven by the control experiment in which the same quantity of the same normal serum manifests no action on an equal quantity of the same leucocyte suspension. The results of such experiments were strikingly positive. An example follows:

A 5 per cent suspension of red corpuscles 1 c.c. + heated serum 0.05 c.c.; place at 37° C. for one hour; centrifugate.

Supernatant fluid + leucocytic suspension 0.2 c.c. + normal serum 0.0015 c.c. = leucolysis.

Leucocytic suspension 0.2 c.c. + normal serum 0.0015 c.c. = no leucolysis.

Reversing this experiment, i. e., saturating the heated serum with leucocytes and using hemolysis as the indicator of reaction, gives results of identical significance.

Leucocytic suspension 1 c.c. + heated serum 0.03 c.c.; place at 37° C. for one hour; centrifugate.

Supernatant fluid + 5 per cent suspension of red corpuscles 1 c.c. + normal serum 0.015 c.c. = hemolysis.

A 5 per cent suspension of red corpuscles 1 c.c. + normal serum 0.015 c.c. = no hemolysis.

The conclusion, therefore, is that the thermostable element of normal leucotoxin is distinct from that of normal hemolysin.

An experiment was made to determine if the thermolabile body necessary for leucotoxic action is the same as that necessary for hemolysis. For this purpose the same method as that in the preceding experiment was employed, and may be outlined as follows:

Leucocytic suspension 0.2 c.c. + heated serum 0.1 c.c. for one hour at 37° C.; centrifugate. Sediment = sensitized leucocytes.

A 5 per cent suspension of red corpuscles 1 c.c. + normal serum 0.03 c.c., for one hour at 37° C.; centrifugate.

Supernatant fluid + sensitized leucocytes = leucolysis.

The result might be interpreted as indicating the non-identity of the complement fraction of hemolysin and leucotoxin but it is not final, for even if it were the same, all the complement need not necessarily be bound when the serum is exposed to an erythrocytic suspension. If the result had been no leucolysis, it would have been positive evidence of the identity of the complemental bodies, but the results as found are ambiguous and throw no light upon the question at issue.

Having shown that normal leucotoxins are not the same bodies as the normal hemolysins, the next experiment was to determine whether or not they are specific, or, in other words, is there but one leucotoxin responsible for all the positive results shown in Table 1, or are there a number of different leucotoxins each responsible for leucolysis in one and only one serum-leucocyte combination? The problem can be investigated best by choosing a serum exerting a toxic action on the leucocytes of more than one species, as chicken serum on dog and guinea-pig leucocytes, and determining by means of absorption experiments whether it is the same substance in the serum that causes both leucolyses. If all the dog leucotoxin be removed from a given quantity of chicken serum by saturation with dog white corpuscles and the fluid obtained by centrifugation is still capable of destroying guinea-pig leucocytes, and the reverse likewise obtains, it is evident that the serum contains at least two distinct leucotoxins, one for dog and one for guinea-pig corpuscles, and consequently we would be justified in holding that leucotoxins are truly specific. Such, indeed, is the case, as shown by these experiments.

Dog leucocytic suspension 1 c.c. + chicken serum 0.2 c.c.

Place at 37° C. for 1 hour. Centrifuge.

Supernatant fluid + dog leucocytic suspension 0.2 c.c. = *no leucolysis*.



Dog leucocytic suspension 1 c.c. + chicken serum 0.2 c.c.

Place at 37° C. for one hour. Centrifuge.

Supernatant fluid + guinea-pig leucocytic suspension 0.2 c.c. = *leucolysis*.

Guinea-pig leucocytic suspension 1 c.c. + chicken serum 0.2 c.c.

Place at 37° C. for one hour. Centrifuge.

Supernatant fluid + guinea-pig leucocytic suspension 0.2 c.c. = *no leucolysis*.

Guinea-pig leucocytic suspension 1 c.c. + chicken serum 0.2 c.c.

Place at 37° C. for one hour. Centrifuge.

Supernatant fluid + dog leucocytic suspension 0.2 c.c. = *leucolysis*.

It has been shown that leucotoxin as a whole is bound by leucocytes, but the question was left open as to which of the constituent fractions is bound by the white corpuscles. To settle this point a quantity of normal serum so small that only the complemental fraction was present was treated with leucocytes. After centrifugation the supernatant fluid was added to a quantity of "sensitized" leucocytes (leucocytes + amboceptor [heated serum]) whereupon leucotoxic action took place, showing that the complemental body had not been removed from the normal serum by the leucocytes.

Leucocytic suspension No. 1, 0.2 c.c. + normal serum 0.0015 c.c.

Expose to 37° C. for one hour; centrifugate and remove fluid.

Leucocytic suspension No. 2, 0.2 c.c. + heated serum 0.1 c.c.

Expose to 37° C. for one hour.

Add centrifugal fluid, and incubate = *leucolysis*.

Leucocytic suspension No. 3, 0.2 c.c. + normal serum 0.0015 c.c. incubated for one hour = *no leucolysis*.

Since the thermolabile fraction is not bound by the normal susceptible cells, it must be the thermostabile body that unites with them, and indeed, such can be demonstrated to be the case, for if leucocytes be exposed to heated serum for an hour, removed by centrifugation, and then exposed to a quantity of unheated serum so small that no fixative is present, leucolysis occurs, showing that the amboceptor had been fixed by the leucocytes.

Leucocytic suspension 1 c.c. + heated serum 1 c.c.

Place at 37° C. for one hour; centrifugate.

Sediment + normal serum 0.0015 c.c. = *leucolysis*.

Leucocytic suspension 0.2 c.c. + normal serum 0.0015 c.c. = *no leucolysis*.

It is of considerable interest to know if there are bodies in the serum of animals capable of neutralizing or preventing the action of the leucotoxins in foreign sera upon the leucocytes of that animal. To investigate this possibility equal quantities of human and of dog serum (0.2 c.c. each) were added to a suspension of dog leucocytes and incu-

bated at  $37^{\circ}$  C. for one hour. At the end of that period it was found that leucolysis had occurred. It was realized that this did not afford absolute proof of the non-occurrence of normal antibodies, since it might be that the affinity of the leucotoxin for the cells is stronger than for the antileucotoxin so that when brought in contact with both leucocyte and antibody the poison unites with the former and the presence of the latter is not appreciated. This error was avoided by leaving the human and dog sera in contact with each other for one hour at  $37^{\circ}$  C. before adding the dog leucocytes to the mixture. At the same time a possible quantitative factor was tested for by employing increasing amounts of dog serum. In no case was leucolysis prevented, showing that normal dog serum, at least, contains no antibody for normal human leucotoxin.

TABLE 4.  
ABSENCE OF NORMAL ANTIBODY FOR LEUCOCYTOTOXIN.

Dog Serum	Human Serum	Dog Leucocytic Suspension	Result
...	0.02 c.c.	0.2 c.c.	Leucolysis
0.02	0.02	0.2	"
0.05	0.02	0.2	"
0.1	0.02	0.2	"
0.2	0.02	0.2	"
0.4	0.02	0.2	"

Another important question is how the leucotoxin content of serum is affected by morbid processes in the body, particularly those diseases in which a leucocytosis occurs. For this purpose the sera of a number of patients suffering from such diseases were analyzed quantitatively for leucotoxins by the following method. Dilutions were made and quantities of 0.003, 0.0015, 0.0007, 0.00035 c.c. and in some cases 0.006 c.c. were added to washed dog leucocytes, and the highest dilution showing positive action was taken as the index to the amount of leucotoxin present in the serum. The results are shown in Table 5. While these figures show that the leucotoxic index does not run entirely parallel to the degree of leucocytosis, still it is quite clear that there is some intimate relation between the two, suggesting that leucotoxin either is produced by the hematopoietic tissues or else that it arises from the leucocytes.

It would be interesting to learn whether it is possible to immunize susceptible animals against the leucotoxins of heterologous sera;

further, whether or not normal leucotoxins are the same as the artificial leucotoxins which may be produced by repeated injections of lymphoid tissue.

TABLE 5.  
INFLUENCE OF LEUCOCYTOSIS UPON LEUCOCYTOTOXIC INDEX.

Disease	Leucocytosis	Leucotoxic Index
Malaria.....	None (5,000)	0.003
Pneumonia No. 1.....	14,000	0.0007
Acute artic. rheumatism.....	17,000	0.0007
Pneumonia No. 2.....	21,000	0.0007
Pleurisy No. 1.....	26,000	0.0007+
Pleurisy with effusion No. 2.....	26,000	0.00035+
Pneumonia No. 3.....	28,700	0.0007
Gonorrheal arthritis.....	28,000	0.00035+
Bronchopneumonia.....	51,000	0.00035+
Scarlatina No. 1.....	No count	0.0015
" No. 2.....	"	0.0015
" No. 3.....	"	0.00035+
" No. 4.....	"	0.007
" No. 5.....	"	0.006
" No. 6.....	"	0.00035
Hemophilia.....	"	0.0015
".....	"	0.0015
".....	"	0.0007
Normal.....	.....	{ 0.006 to / 0.003

The results above obtained may be summarized as follows:

Normal leucotoxins have a fairly wide distribution. They consist of a thermostabile specific body and a thermolabile non-specific substance.

They are distinct from the normal hemolysins, have a different distribution, and are present in considerably smaller amounts of serum.

Their action is essentially upon the nuclei of the leucocytes. They affect all types of leucocytes, but the large mononuclear variety is the most susceptible and the polymorphonuclear form the least susceptible to their action.

Normal sera do not appear to contain antibodies to the leucotoxin of foreign sera.

In diseases associated with a leucocytosis there is an increase in the amount of normal leucotoxin in the serum, this increase corresponding more or less to the extent of the leucocytosis.

In conclusion it is perhaps permissible to point out that, in choosing combinations for experimental opsonic work, proper results can be obtained only by avoiding those in which the serum exerts a poisonous action upon the leucocytes.

I wish to thank Professor Ludvig Hektoen here for his valued advice and suggestions in regard to this investigation.

## THE DURATION AND DISAPPEARANCE OF PASSIVE DIPHTHERIC IMMUNITY.\*†

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THE value of passive immunization with antitoxic serum for prophylaxis against diphtheria is now so well appreciated and its use for this purpose so widespread that information concerning the duration and extent of the protection thus conferred is of considerable practical, as well as scientific, importance.

The literature contains several clinical estimates of the approximate duration of this passive immunity. Thus, Rubens<sup>1</sup> places it at about four weeks when a dose of 200 units is employed; Behring<sup>2</sup> from observations made at Heubner's clinic gives a similar figure; Aaser<sup>3</sup> estimates it at three to four weeks; Maag<sup>4</sup> at five weeks; and Sittler<sup>5</sup> at three to five weeks when the child is not exposed, and at 10 to 14 days when in contact with diphtheria patients. Accurate determinations, however, have been limited to the persistence of antitoxin in the serum of injected animals. Passini<sup>6</sup> found that after the injection of 2,000 units into an adult man antitoxin could be demonstrated in the serum for 11 days, while following the injection of 200 units into a goat it could be detected only up to the third day. Mueller<sup>7</sup> working on children found antitoxin present in their serum for from 6 to 37 days after administration, with no constancy. Neither of these investigators made quantitative determinations. Behring, however, after injecting 25,000 units into a goat made quantitative examinations of the amount of antitoxin in each cubic centimeter of serum and found that on the 23d day an appreciable quantity was still present, but did not continue his observations any farther. Kraus and Joachim<sup>8</sup> tested the sera of rabbits and guinea-pigs at intervals of one and 24 hours after the

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<sup>1</sup> *Deutsche med. Wchnschr.*, 1895, 21, p. 758.

<sup>5</sup> *Jahrb. f. Kinderheilk.*, 1906, 44, p. 3.

<sup>2</sup> *Fortschr. d. Med.*, 1897, 15, p. 1.

<sup>6</sup> *Wien. klin. Wchnschr.*, 1896, 9, p. 1111.

<sup>3</sup> *Berl. klin. Wchnschr.*, 1905, 42, p. 38.

<sup>7</sup> *Jahrb. f. Kinderheilk.*, 1897, 44, p. 394.

<sup>4</sup> *Jour. Am. Med. Assoc.*, 1906, 47, p. 158 (abstract).

<sup>8</sup> *Wien. klin. Wchnschr.*, 1903, 16, p. 1389.

injection of antitoxin, but their results vary from 33 per cent to 90 per cent and show no constancy. Bulloch<sup>1</sup> injected 25,000 units subcutaneously into an ass and made a number of quantitative determinations at intervals of from 30 minutes to 126 days. The maximum he found at 24 hours and the final disappearance did not occur until sometime between the 100th and 126th days. J. H. Smith<sup>2</sup> performed a similar experiment using a man as subject. He administered 9,000 units ( $5\frac{1}{3}$  units per c.c. of blood serum) into the subcutaneous tissues and made a series of quantitative determinations of the serum, finding the maximum, reached on the third day, was less than one-fourth of the amount given, and that all trace of it was lost somewhere between the 20th and 27th days. Finally, Bomstein<sup>3</sup> followed the disappearance of injected antitoxin from the blood of the dog and the guinea-pig, finding it entirely gone from the former in 18 days and from the latter in 22 days.

From the above it may be seen that not only have there been no attempts to gauge accurately the extent and duration of the immunity of the body as a whole, but that there is no information as to the relation exhibited between the amount of free antitoxin circulating in the blood stream and the extent of the immunity it affords the subject, and further, whether the disappearance of the somatic immunity (that is, the immunity of the body as a whole, as contrasted with that due to the blood) is synchronous with that of the antitoxin in the blood. It is scarcely necessary to dwell upon the practical value to preventive medicine of definite knowledge upon these points.

To obtain this information the extent of the somatic immunity and the amount of free antitoxin circulating in the blood were determined separately at each 24-hour period in rabbits injected with diphtheria antitoxin. The rabbit was chosen for this investigation partly because no definite work had been previously done with it, but more especially because of its high degree of susceptibility to diphtheria toxin, as determined in a previous study.<sup>4</sup> Gram for gram the susceptibility of the rabbit to diphtheria toxin introduced intraperitoneally is three times as great as that of the guinea-pig. Since but a small excess of

<sup>1</sup> *Jour. Path. and Bact.*, 1898, 5, p. 274.

<sup>2</sup> *Jour. of Hyg.*, 1907, 7, p. 205.

<sup>3</sup> *Centralbl. f. Bakt.*, 1897, 22, p. 587.

<sup>4</sup> Goodman, *Jour. Infect. Dis.*, 1907, 4, p. 500.

toxin suffices to kill, the death of the animal affords a biological indicator of the neutral point in the toxin-antitoxin titration that is at once delicate and unambiguous. Intraperitoneal injections were employed exclusively with the rabbits, the advantage being that the animal is about three times as susceptible to this mode as to subcutaneous injection and equally as susceptible as to intravenous administration<sup>1</sup> while the intraperitoneal is more conveniently performed than the latter method, and finally the antitoxin is much more rapidly absorbed after intraperitoneal than after subcutaneous injection.<sup>2</sup>

To determine the curve of the general passive immunity, rabbits were injected with five units of antitoxin per kilo of body weight (0.1 unit per gram of blood) and the amount of diphtheria toxin necessary to cause acute death in four or five days determined for each 24-hour interval until the immunity had fallen to the amount normal for the species. In this way the fraction of the original passive immunity persisting at each of these intervals was ascertained and was calculated in per cent of the original injection. In a few of the last stages a larger quantity of antitoxin than the above was necessary because of the minuteness of the fraction of the original amount persisting. The details of the experiment are shown in Table 1.

To determine the proportion of injected antitoxin persisting in the blood at each of these intervals, rabbits were injected with four units of antitoxin per gram of blood (estimating the blood as  $\frac{1}{20}$  of the body weight according to Landois), and at the expiration of the period for which the test was intended the animal was bled, with aseptic precautions, from the ear vein or from the heart according to the amount of blood desired, into a sterile test-tube marked at the level of the desired volume of blood. The blood was then placed in the thermostat at 37° C. for one hour to hasten coagulation, and after the serum had separated sufficient diphtheria toxin was added to neutralize the original content of antitoxin to the desired point, the mixture being made up to 5 c.c. with sterile salt solution if less than that volume, and injected subcutaneously into a guinea-pig without further standing since Morgenroth<sup>3</sup> and Otto and Sachs<sup>4</sup> have shown

<sup>1</sup> Goodman, *Jour. Infect. Dis.*, 1907, 4, p. 509.

<sup>2</sup> Smith, J. H., *Jour. of Hyg.*, 1907, 7, p. 205.

<sup>3</sup> *Ztschr. f. Hyg.*, 1904, 48, p. 177; *Berl. klin. Wchnschr.*, 1904, 41, p. 526.

<sup>4</sup> *Ztschr. f. Exp. Path. u. Ther.*, 1906, 3, p. 19.

TABLE I.  
DECREASE IN PASSIVE IMMUNITY OF RABBIT.

No. Days. Interval be- tween Injections	Weight of Rabbit	Units Anti- toxin Injected	Guinea-Pig M. L. D. Toxin Given	Percentage Neutralization of Antitoxin	No. Days Rabbit Survived
1	2,080 1,620 1,850	10.40 8.10 9.25	520. 270. 231.25	50. 33.33 25.	$\frac{1}{2}$ $4\frac{1}{2}$ †
2	2,220 2,400 2,030	11.10 12.00 10.15	370. 384. 304.5	33.33 32. 30.	$3\frac{1}{2}$ 4 †
3	1,955	9.78	293.4	30.	5
4	1,705 1,490 2,040	8.52 7.45 10.20	238.55 178.8 204.	28. 24. 20.	$2\frac{1}{2}$ $4\frac{1}{2}$ $7\frac{1}{2}$
5	1,330 1,720 2,140	6.65 8.60 10.70	123. 137.6 160.5	20. 16. 15.	3 $4\frac{1}{2}$ $5\frac{1}{2}$
6	1,660 1,925	8.30 9.62	124.5 134.70	15. 14.	$3\frac{1}{2}$ 5
7	2,300 2,085	11.95 10.42	155.35 125.05	13. 12.	4 $4\frac{1}{2}$
8	1,535 1,600 1,710	7.68 8.45 8.52	84.50 84.50 76.70	11. 10. 9.	$3\frac{1}{2}$ 3 $4\frac{1}{2}$
9	2,080	10.40	83.20	8.	$4\frac{1}{2}$
10	2,105 1,385	10.52 6.92	84.15 48.45	8. 7.	4 $6\frac{1}{2}$
11	1,990 2,200	9.95 11.00	74.65 77.00	7.5 7.	5 †
12	1,680	8.40	58.8	7.	$5\frac{1}{2}$
13	1,630	8.15	57.05	6.5	4
14	1,275 2,410 1,725	6.38 12.05 8.62	44.65 72.30 47.40	6.5 6. 5.5	$2\frac{1}{2}$ $3\frac{1}{2}$ 4
15	2,265 2,330	11.32 11.65	56.60 46.60	5. 4.	3 $4\frac{1}{2}$
16	1,800	9.00	27.	3.	5
17	1,730 2,085 2,760	8.65 10.42 13.80	21.65 22.90 27.6	2.5 2.2 2.	$3\frac{1}{2}$ 5 $7\frac{1}{2}$
18	1,890 1,900	9.45 9.50	18.9 17.1	2. 1.8	$3\frac{1}{2}$ $4\frac{1}{2}$
19	1,840 1,720	9.20 8.60	13.8 11.20	1.5 1.3	3 4
20	1,205	12.05†	12.05	1.	5
21	1,815 1,285	16.15† 12.85†	14.55 8.95	0.8 0.7	3 $4\frac{1}{2}$
22	1,560 1,605	15.60† 16.05†	9.30 8.00	0.6 0.5	2 $5\frac{1}{2}$
23	1,130 1,285	22.60† 25.70†	9.05 6.40	0.4 0.25	$2\frac{1}{2}$ 4
24	1,310	26.20†	2.60*	0.1	†

\* = more than the intraperitoneal M. L. D. † Double quantities. ‡ Quadruple quantities.

Days after Injection	SUMMARY.	Residue of Immunity
1		33 $\frac{1}{2}$ per cent
2		32
3		30
4		24
5		16
6		14
7		12
8		9
9		8
10		8
11		7.5
12		7
13		6.5
14		5.5
15		4
16		3
17		2.2
18		1.8
19		1.3
20		1
21		0.7
22		0.5
23		0.25
24		0.00

this to be unnecessary in the case of subcutaneous injections of toxin-antitoxin mixtures. Death of the guinea-pig on the fourth or fifth day was taken to indicate exact neutralization of the antitoxin and therefore showing the amount thereof still in the blood. The above-described method of collecting the blood was devised in order to obviate the necessity of making correction calculations for the ratio of serum to normal blood (practically none of the antitoxin is in the formed elements of the blood according to Dzerjgowsky,<sup>1</sup> since by this method all the serum obtainable from the given amount of blood was collected and it is quite inconsequential, so far as the accuracy of the experiment is concerned, whether 1 c.c. of blood yields  $\frac{2}{3}$  c.c. of serum (Jørgensen and Madsen) or only  $\frac{1}{2}$  c.c. of serum (Kraus and Joachim) or an inconstant quantity. In Table 2 are shown the minutiae of this experiment.

The results of the two experiments are recorded graphically in Chart 1. It will be seen that the fall in the passive immunity is quite rapid, during the initial two or three days comparatively gradual, but very abrupt during the ensuing week, the final disappearance being prolonged disproportionately and not occurring for some 3 $\frac{1}{2}$

<sup>1</sup> *Acad. des Sci. Biol. de St. Pet.*, 1897, 5, p. 123.



TABLE 2.  
AMOUNT OF FREE ANTITOXIN IN BLOOD.

No. Days Interval before Bleeding	Weight of Rabbit	Units Anti- toxin Injected	Cubic Centi- meters Rabbit's Blood Used	Guinea-pig M. L. D. Toxin Injected	Percentage Neutralization of Antitoxin	No. Days Guinea-pig Survived
1	2,200	440	0.05	10.0	50	1 $\frac{1}{2}$
	1,680	336	0.05	5.0	25	1 $\frac{1}{2}$
	1,810	362	0.05	4.0	20	4 $\frac{1}{2}$
2	1,495	299	0.05	4.0	20	3 $\frac{1}{2}$
	1,840	368	0.05	3.9	19.5	4 $\frac{1}{2}$
3	2,320	464	0.05	3.8	19	3
	2,170	434	0.05	3.6	18	4
4	1,905	381	0.05	3.4	17	2 $\frac{1}{2}$
	1,290	258	0.05	3.2	16	4 $\frac{1}{2}$
	1,660	332	0.05	3.0	15	6 $\frac{1}{2}$
5	1,710	342	0.05	3.0	15	1 $\frac{1}{2}$
	2,440	488	0.1	5.0	12.5	5
	2,225	445	0.05	2.0	10	6 $\frac{1}{2}$
6	1,700	340	0.05	2.0	10	3 $\frac{1}{2}$
	2,150	430	0.1	3.0	9	4
7	2,080	416	0.1	3.6	9	1 $\frac{1}{2}$
	2,200	440	0.1	2.0	5	4 $\frac{1}{2}$
	2,110	422	0.25	4.0	4	6 $\frac{1}{2}$
8	1,770	354	0.3	3.6	3	4
9	1,660	332	0.25	2.0	2	3
	1,800	360	0.5	3.4	1.7	5
	1,410	282	0.5	3.0	1.5	5 $\frac{1}{2}$
10	1,640	328	0.5	3.0	1.5	3
	2,080	416	0.5	2.6	1.3	4 $\frac{1}{2}$
11	2,120	424	0.5	2.4	1.2	2 $\frac{1}{2}$
	2,020	404	0.55	2.0	0.9	5 $\frac{1}{2}$
12	1,670	334	1.44	4.0	0.7	3 $\frac{1}{2}$
	1,755	351	1.00	2.	0.5	2 $\frac{1}{2}$
	2,205	441	1.25	2.	0.4	4 $\frac{1}{2}$
13	1,950	390	1.67	2.	0.3	2 $\frac{1}{2}$
	1,770	354	2.5	2.	0.2	4 $\frac{1}{2}$
	1,290	258	5.	2.	0.1	5 $\frac{1}{2}$
14	1,400	280	5.	2.	0.1	2
	1,970	394	7.5	1.5	0.05	4 $\frac{1}{2}$ *
	1,550	310	10 †	2	0.025	16?
15	1,380	276	10 †	2	0.025	12?

\* Ulcer.

† Double quantity of antitoxin used.

## SUMMARY.

No. Days after Injection	Residue of Anti- toxin in Blood
1.....	20 per cent
2.....	19.5
3.....	18
4.....	16
5.....	12.5
6.....	9
7.....	5
8.....	3
9.....	1.7
10.....	1.3

11.....	.9
12.....	.4
13.....	.1
14.....	.05
15.....	.00

weeks. The fall in the antitoxin content of the blood is similar in general to that in the body protection, but presents two significant points of difference. In the first place it will be observed that relatively to the amount of antitoxin administered the general immunity

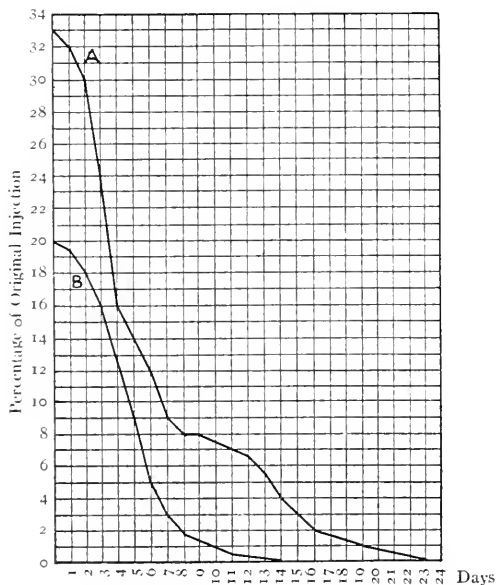


CHART 1.—A, Somatic Immunity; B, Antitoxin in Blood.

is always appreciably higher than the quantity of antitoxin free in the blood would lead one to expect, and in the second place the former persists considerably longer than the latter (nine days). The importance of these points is that they show that the amount of antitoxin circulating in the blood does not fully gauge the degree of protection conferred by an injection of the protective serum, the latter always being higher, the difference ranging, in terms of

the quantity administered, from 13 per cent on the first to 3.5 per cent on the fifth day, and secondly they show that the subject has a fair degree of protection for some time after all detectable antitoxin has disappeared from the blood.

It is of interest, at this point, to compare the foregoing results with those that others have obtained for the rabbit with tetanus antitoxin. Ransom<sup>1</sup> and Knorr<sup>2</sup> made quantitative determinations of the antitoxin content of the blood at several periods during the sojourn of tetanus antitoxin in the rabbit, and have constructed curves

<sup>1</sup> *Jour. Path. and Bact.*, 1900, 6, p. 180.

<sup>2</sup> Quoted by Ransom.

illustrating the hemic immunity. As is to be expected in the case of so different a substance, these curves differ in their details from Curve B, Chart 1, but they nevertheless correspond to it in general character, and may therefore be regarded as in a way confirmatory of the general accuracy of the results obtained here with the diphtheria antitoxin. Tizzoni and Catani<sup>1</sup> also have studied the persistence of tetanus antitoxin in the rabbit, using antisera obtained from the horse, dog, and rabbit. When that of the horse was used, no trace of the antitoxin remained after 15 days, which by an interesting coincidence is the exact duration of diphtheria antitoxin in the rabbit, as established above.

The obvious explanation of the discrepancy between the results shown in Table 1 and those in Table 2 would be that there occurs a storing-up of some of the injected antitoxin by the body tissues. The literature, however, appears to argue against such a view. Both Behring from observations made upon the goat and Bulloch from studies on the ass consider that almost all the antitoxin rapidly enters the serum as they found most of it there a few hours after the injection; Kraus and Joachim observed that the organs of normal rabbits have no power to combine with diphtheria antitoxin *in vitro*, and Bomstein states that in immunized guinea-pigs the organs contain either no antitoxin or no more than can be accounted for by their blood content. Kraus and Eisenberg<sup>2</sup> and Kraus and Joachim have shown that diphtheria antitoxin is capable of producing no antibody to itself. Careful consideration of these points, however, must fail to convince that the above-mentioned hypothesis is thereby excluded, since, as far as the data of Behring and of Bulloch are concerned, it might well be that the serotropism is but a preliminary phenomenon and that after accumulating in the serum a part of the antitoxin passes thence into the tissues. Further, the observations of Kraus and Joachim by no means exclude the possibility that such a storing-up of the antitoxin does occur in the living body, and finally Bomstein's report, although apparently ruling out such a process, is far from being clear or definite. While giving no experimental data on the point, he states that the liver, lungs, and spleen contain only as much antitoxin as can be attributed to their blood content. A mom-

<sup>1</sup> *Berl. klin. Wchschr.*, 1896, p. 1186.

<sup>2</sup> *Centrabl. j. Bakt.*, 1902, 31, Orig., p. 208.

ent's reflection, however, will show that there is a palpable miscalculation involved in this statement. At the time of his test, the fourth day, according to him the blood of the guinea-pig contains about one-fifth of the amount originally injected, so that even taking into account the excess of the somatic over the blood immunity as shown above, a simple computation will demonstrate that the blood as compared to the other tissues contains gram for gram so great an excess of antitoxin that no matter how thoroughly the guinea-pig was bled before the experiment, the residue of blood in organs as vascular as these would contain much more antitoxin than the viscera themselves could possibly have. The same objection applies to his statement that no antitoxin at all could be demonstrated in other viscera. In view, therefore, of the strong presumptive evidence that an accumulation of antitoxin occurs in the tissues, it was thought advisable to repeat upon the rabbit experiments somewhat similar to those of Bomstein upon the guinea-pig. It is evident, however, that as long as any quantity of antitoxin is circulating in the blood, such an examination of the tissues is useless, because it is impossible to gauge the quantity of blood in the tissue examined, and consequently one is unable to say what moiety of the toxin-neutralizing power is due to antitoxin in the tissue and not to that in the blood contained in the viscus. Fortunately, this difficulty can be obviated by choosing for the experiment a period at which all antitoxin has disappeared from the blood, but not from the body, such, for instance, as the 15th day, which shows the highest somatic immunity of the periods following the disappearance of the antitoxin from the blood. A rabbit weighing 990 grams was given 1,000 units intraperitoneally and killed after the lapse of 15 days. At this period we would expect to find about 4 per cent of the immunity afforded by the original injection, but, as will be shown later, the increased size of the injection necessitates raising this figure to somewhat over 5 per cent, so that if uniformly distributed throughout the animal's body each gram of tissue should be able to neutralize at least five guinea-pig minimum lethal doses of diphtheria toxin, the actual neutralizing power being still higher. Pieces of liver, spleen, brain, muscle, and connective tissue were removed, pressed out through sterile cheese-cloth, the liquid mixed with 3 M. L. D. of toxin and injected into a guinea-pig subcutaneously

after the volume had been made up to 4 c.c. with normal salt solution. About 1.5 grams of each tissue were used. To reduce the danger of contamination these pieces were not weighed each time but were made to correspond in size, as nearly as could be judged, with weighed pieces removed from another animal. The first three tissues were injected separately, the last two together into one guinea-pig. All four animals died acutely in not over three days and upon postmortem examination showed the typical signs of diphtheric intoxication. The evidence afforded by the experiment appears to demonstrate that antitoxin is not stored as such by the tissues, and while it merely corroborates the evidence of the previous investigators, it does so in a perhaps somewhat less equivocal fashion. Regarding the question of the discrepancy between the blood and the body immunity, it leaves us wholly at sea. We see that the blood does not contain all the antitoxin in the body and therefore the excess is in the tissues, but then how are we to account for the inability of the tissues to neutralize toxin in the test-tube? It might be suggested that the excess of antitoxin is stored in the tissues in some form so altered that it is no longer capable of binding toxin when added to the latter *in vitro*, but if so it would not be able to neutralize toxin *in vivo*, and yet such is clearly what it does. The only explanation occurring to me that overcomes this objection is one which assumes that within the living body the cells storing this altered antitoxin respond to the stimulus of the presence of free toxin in the body, as evinced possibly by injury to themselves, by reconverting the antitoxin to its original and chemically active form in order to protect themselves against further damage by the toxin. As to the exact process involved, perhaps the simplest to comprehend would be the occurrence of a change in solubility. When the soluble circulating antitoxin is taken up by the tissues it is precipitated, by an out-salting it may be, and stored in this insoluble form until in response to the stimulus afforded by the presence of toxin, the cells reverse their physicochemical conditions and the antitoxin goes into solution once more. While, unfortunately, such a conception cannot be put to experimental proof, it seems to be the only explanation not incompatible with the facts already determined, and, moreover, is supported by analogy with the similar conception

of the mechanism of the storing and release of cumulative substances like digitalis.

The next question arising is the manner in which the animal finally rids itself of injected antitoxin. Behring, obtaining evidence of the presence of antitoxin in the milk and urine of passively immunized goats, held that it is eliminated exclusively by the secretions, while, on the other hand, Bulloch, finding no antitoxin in the urine of the ass at a time when the blood content was rapidly decreasing, considers that excretion by the kidneys is not concerned in the process of elimination, and Bomstein, observing that for three or four days the urine of guinea-pigs contained but  $\frac{1}{300}$  of the amount of antitoxin then present in the blood, and the viscera, as noted earlier, little or none, suggests that the disappearance is due to some chemical change. These views are thus diametrically opposed, and the importance of the question, it was felt, justified attempting a quantitative examination of the urine of passively immunized rabbits for antitoxin, especially since elimination by the kidneys has been demonstrated (although not quantitatively) in the case of other antitoxins, that of tetanus by Vagedes<sup>1</sup> and by Behring,<sup>2</sup> and that of *B. pyocyaneus* by Bouchard.<sup>3</sup> It may be of interest, further, to note that Ehrlich and Wassermann<sup>4</sup> estimate that the milk contains from  $\frac{1}{15}$  to  $\frac{1}{30}$  of the blood's content of antitoxin in actively immunized animals.

The technic for this experiment was similar to that of the blood determination already described. Upon the proper day urine was drawn from animals in use for that test by means of a sterile catheter, when necessary levying upon two or more animals to make up the required volume of urine. To the latter was next added diphtheria toxin in such quantity as to neutralize to the desired degree the original blood content of antitoxin, that is, the animal being given four units of antitoxin per cubic centimeter of blood, the urine was titrated to learn what fraction of the four units was present in each cubic centimeter of urine at the particular period under consideration. The toxin-urine

<sup>1</sup> *Ztschr. f. Hyg.*, 1895, 20, p. 205.

<sup>2</sup> *Infektion u. Desinfektion*, Leipzig, 1894, p. 183.

<sup>3</sup> *Compt. rend. Acad. Sci.*, 1888, 106, p. 1582.

<sup>4</sup> *Ztschr. f. Hyg.*, 1894, 18, p. 248.

mixtures after being diluted with normal salt solution up to 5 c.c., if below that volume, were injected subcutaneously into guinea-pigs as in the former experiment. In order to economize, tests were omitted on several of the days, but as these did not include the final day, a reasonably accurate idea of the curve of elimination by this channel is afforded.

TABLE 3.  
EXCRETION OF ANTITOXIN BY THE URINE.

Days Interval between Injection and Catheterization	Cubic Centi- meters Urine Injected	M. L. D. Toxin Added	Percentage Neutraliza- tion	No. Days Guinea-pig Survived
3	0.25	2.0	2.	1
	0.5	2.0	1.	2½
	1.	2.6	0.65	4½
	1.	2.0	0.5	6½
5	0.4	2.4	1.5	3½
	0.5	2.8	1.4	4½
	0.5	2.0	1.	6½
	1.	2.0	0.5	7
7	0.5	2.8	1.4	4½
9	0.5	2.8	1.4	1½
	0.5	2.0	1.	2½
	1.	2.0	0.5	5
13	4.	2.4	0.25	3
	5.	2.0	0.10	4
14*	5.	2.	0.05	2½

\* Eight units per cubic centimeter blood injected.

The results are shown in Table 3 and may be summarized thus:

SUMMARY.

3d day.....	0.65 per cent
5th ".....	1.4
7th ".....	1.4
9th ".....	0.5
13th ".....	0.1
14th ".....	below 0.05 per cent

When compared with those in the blood these quantities are seen to be quite insignificant for the first week or so, thus corresponding in general with Bomstein's results, but then, while still decreasing absolutely, the quantity in the urine increases so rapidly relatively to that in the blood that upon the 13th day just preceding its disappearance from the urine, it actually equals the blood's content. Considering it from this standpoint, however, is not justifiable, for it is evident that the only proper way to judge the importance of the rôle played by the kidneys in ridding the system of antitoxin is to

compare the urinary finding with the decrease in the blood content for the same period, as in this way the extent to which other agencies participate in the process is shown. Another fallacy, although of a minor character, is that involved in the comparison of the urinary content with the actual amount of the decrease in the blood's content, for since the volume of urine secreted in 24 hours under ordinary conditions approximates only about one-fourth that of the blood, substances passing from the latter into the urine will appear to be increased fourfold when equal volumes of the two fluids are compared. Consequently, in order to obtain a correct conception of this process, the urinary findings must be compared with four times the decrease in antitoxin content of the blood for the same period.

In Table 4 there is compiled for the purpose of comparison the quantity of antitoxin present in the blood on each day, the decrease

TABLE 4.

Days after Injection	Blood's Content Percentage	Blood's Loss from Previous Day	Urine if Complete	Percentage Actually Found	Approximate Proportion of Complete
1	20.	...	...		
2	10.5	0.5	2.		
3	18.	1.5	6.	0.65	11
4	16.	2.	8.		
5	12.5	3.5	14.	1.4	10
6	9.	3.5	14.		
7	5.	4.	16.	1.4	9
8	3.	2.	8.		
9	1.7	1.3	5.2	0.5	10
10	1.3	0.4	1.6		
11	0.9	0.4	1.6		
12	0.4	0.5	2.		
13	0.1	0.3	1.2	0.1	8½
14	0.05	0.05	0.2	0.00	...
15	...	0.05	0.2		

therein for each period, the theoretical complete urinary content, i. e., the amount of antitoxin that would be present in each cubic centimeter of urine if all disappearing from the blood each day were to go into the urine, the actual quantities found in the urine and the proportion borne by the latter to the theoretical complete urinary content. A general survey of the relations of these factors to each other is facilitated by reference to Chart 2 in which these points are presented graphically. The important fact to be noted is that the proportion borne by the actual urinary content of antitoxin and the theoretical complete content varies between 8 and 11 per cent, with a rough average of 10 per cent; in other words, approximately 10



per cent only of the decrease in the antitoxin content of the blood goes into the urine, results differing materially from the observations recorded in the literature as cited above. We are, then, justified in concluding that while elimination of the antitoxin by the kidneys is undoubtedly a factor in the mechanism of its disappearance from the body, it is of minor importance and merely subordinate to some more extensive process. What this process may be we can merely

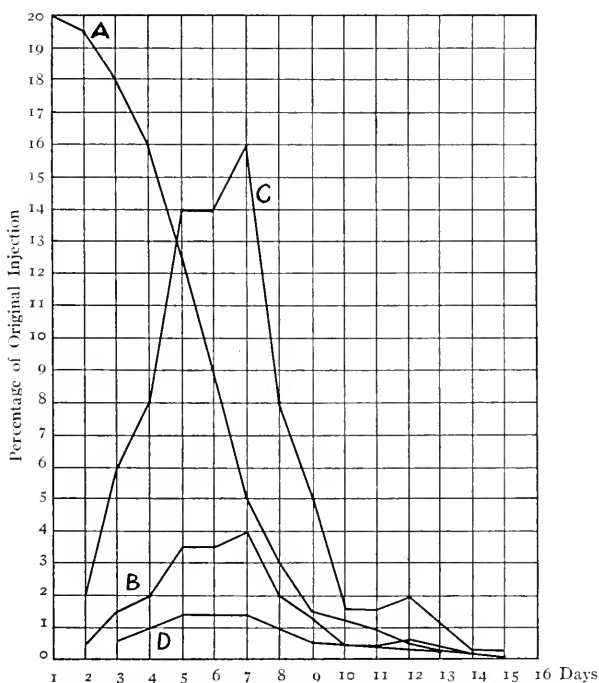


CHART 2.—A=Antitoxin content of Blood; B=Decrease in same from previous day; C=Antitoxin content of urine if all of B were to appear in it; D=Antitoxin content of urine as found. Cf. Table 4

surmise at present, but it is highly probable that it is intimately concerned with the peculiar phenomenon of the excess and longer duration of the somatic over the hemic immunity, and it seems not unlikely that the remaining 90 per cent of antitoxin disappearing from the blood is converted into the form in which it is stored. The daily quantitative discrepancy exhibited between the amount missing from the blood and unaccounted for by the urine content with that of the excess of the somatic over the hemic antitoxin content does not by

any means exclude this possibility, as the rate of disappearance of the chemically inactive form of the antitoxin cannot be determined and may very well be quite different from that of the elimination of the active form from the blood. Concerning the elimination of the chemically inactive form stored in the tissues, the process cannot be initiated by reconversion to the active form, for in that case the antitoxin could be detected in the blood, the rate of this excretion being too rapid to admit of the possibility of the active antibody passing into the blood in quantities too small for detection by biologic methods. This point together with the paucity of antitoxin in the urine in the later stages was considered as sufficient ground for omitting an examination of the urine after the second week. The only alternative, then, is that the tissue cells throw off the antitoxin into the blood in the inactive form in which they have held it and it is then excreted, or else, after storing it for a short time, the cells alter its chemical identity in some way, perhaps, by utilizing it as a food.

It has been shown in an earlier paper<sup>1</sup> that in certain animals age is a very important factor in determining the reaction of the body tissues to diphtheria toxin. In view of the fact that antitoxin is used chiefly upon children, the question whether this phenomenon applies to the antitoxin as well as to the toxin, has considerable interest. The matter was investigated by repeating certain of the titrations for somatic immunity upon young rabbits, the original series having consisted wholly of grown animals. Comparison of the findings (see Table 5) shows that in the rabbit, at least, age is practically of no consequence as a factor in determining the rate of disappearance of injected antitoxin.

Another problem of some practical bearing in connection with the prophylactic use of antidiphtheric serum is how variations in the amount injected affects the extent and duration of the immunity afforded. It is true Behring states that its persistence in the blood of the goat is but slightly increased with the amount given, and Mueller found that in children there was apparently no uniformity between the quantity injected and the duration of the antitoxin in the blood, but inasmuch as neither of these investigators made

<sup>1</sup> Goodman, *Jour. Infect. Dis.*, 1927, 4, p. 500.

TABLE 5.  
INFLUENCE OF AGE UPON ELIMINATION OF ANTITOXIN.

Days Interval	Series	Weight of Rabbit	Units Injected	Guinea-pig M. L. D. of Toxin	Percentage of Neutralization	No. Days Survived
5	Young	740	3.7	66.6	18	3
		640	3.2	51.2	16	5
	Adult	1,720	8.6	137.6	16	4½
		2,140	10.7	160.5	15	5½
7	Young	720	3.6	50.4	12	3½
		2,390	11.05	155.35	13	4
	Adult	2,085	10.42	125.05	12	4½

systematic quantitative determinations, and further, since, as shown above, the amount of antitoxin in the blood is not a criterion of the extent of the general immunity, the following experiment was undertaken. A series of rabbits were given antitoxin injections in amounts of 1, 2, 5, 10, 20, 50, and 100 times the quantity used in determining the somatic immunity (five units per kilo of body weight), and after the lapse of 15 days the residue of the original immunity persisting in each case was determined by biologic titration as before. The results of the experiment recorded in Table 6 seem to show clearly,

TABLE 6.  
RATIO BETWEEN AMOUNT OF ANTITOXIN INJECTED AND RATE OF ELIMINATION.

Units per c.c. Blood	Weight of Rabbit	Units Anti-toxin Injected	Guinea-pig M. L. D. of Toxin Injected	Percentage of Neutralization of Antitoxin	No. Days Rabbit Survived
5	1,000	5.	20.	4.	4½
10	1,320	13.2	52.8	4.	5½
	945	9.45	37.8	4.	4
25	1,610	40.25	161.0	4.	4½
50	1,830	91.5	411.75	4.5	2½
	1,515	75.75	318.15	4.2	5
	1,170	58.5	234.	4.	6½
100	1,700	170.	782.	4.6	3
	980	98.	431.2	4.4	5
250	1,205	301.25	1,506.25	5.	3½
	1,265	316.25	1,486.4	4.7	4½
	1,445	361.25	1,625.6	4.5	6
500	1,025	512.5	2,613.75	5.1	3
	920	460.	2,254.	4.9	5½
	1,000	545.	2,561.5	4.7	7½

that upon increasing the amount of antitoxin given, the quantity eliminated is increased *pari passu*; indeed, not until the amount injected has been increased tenfold is there the slightest appreciable

change in the rate of disappearance, and even with one hundred times the amount of the original injection this rate is so nearly the same that on the 15th day there is a difference of but a trifle over 1 per cent in the ratios borne by the residues in the body to the original injections. While, therefore, at any given period the actual extent of the immunity is greater with larger injections of antitoxin, since the elimination is correspondingly accelerated, the duration of the immunity is scarcely lengthened. A short experiment was performed to verify this deduction. Reference to Table 1 shows that with an injection of 20 units of antitoxin per kilo of weight (0.4 units per gram of blood) no exaltation of the natural immunity could be demonstrated after the 23d day. Another animal was now given an injection of 500 units per kilo of weight (10 units per gram of blood), or 25 times the previous quantity, and tested as before upon the 26th day, when it was found that antitoxin was present, if at all, in a quantity of less than  $\frac{1}{20}$  of 1 per cent.

Objections may be raised to the results obtained here on the ground that the L + dose of toxin rather than the M. L. D. should have been used in testing for antitoxin, as it is more accurate and reliable because less affected by the proportion of toxoids in the filtrate. This, of course is quite true, but in this particular study I do not think accuracy was sacrificed by the choice of the quantity-standard of toxin. In the first place, in all the above experiments (excepting only those recorded in Table 5) the same toxin was employed, and it seems but reasonable to assume that the correction curve that could be applied to it, because of the gradually increasing toxoid content, is less complicated and more uniform than that applying to a number of toxins employed at the L + dose, if that had been the case, for the proportion of toxoid in the binding-units with affinities between the L° and the L + doses likewise is different in each filtrate. In the second place, a double-standard value for the neutralization is avoided. While the L + dose could have been used in ascertaining the antitoxin content of the blood, since that was tested upon guinea-pigs, it naturally could not have been used in determining the somatic immunity, for the L + is purely a guinea-pig dose and cannot be used upon rabbits because the amount of toxin between the L° and L + doses, while fatal to guinea-pigs, is by no means so to rabbits. It would therefore be necessary to establish

a "rabbit L + dose" for the somatic immunity titration while using a "guinea-pig L + dose" for the hemic immunity, and we do not know in such a case that we are employing comparable standards. On the other hand, the use solely of the guinea-pig M. L. D., as in these experiments, obviates this uncertainty and makes it possible to consider everything in terms of guinea-pig resistance, a surprisingly constant quantity. This security, I believe, is of great importance in these experiments because of the necessity of comparing reaction in two different species of animals. Then, with the M. L. D. very much smaller quantities of antitoxin can be detected than with the L + dose. This not merely permits of following the history of the antitoxin more closely, but removes the necessity of injecting amounts of both antitoxin so large that they would, it is likely, provoke extraneous reactions on the part of the animal tending to obscure the point at issue, and not improbably interfere seriously with the accuracy of the experiment. Finally, to learn how great a difference, if any, might occur between titrations based on the L + dose and those on the M. L. D. a series of parallel determinations with the former were made upon the antitoxin content of the blood. The results obtained, shown in Table 7, are not exactly the same, it is true, as those secured by means of the M. L. D. (see Table 8 for the comparison), but they are close enough

TABLE 7.  
TITRATION WITH L+ DOSE.

Days Interval	Weight of Rabbit	Units Antitoxin Injected	Units per Gram Weight	Cubic Centimeters Blood Added to L+ Dose	Percentage of Neutralization	No. Days Guinea-pig Survived
2	1,580	632	8	0.5	25	1½
	1,760	704	8	0.625	20	4
	1,790	706	8	0.7	18	+
4	1,820	728	8	0.78	16	1½
	1,860	744	8	0.89	14	3½
	1,800	706	8	0.96	13	5½
7	2,000	800	8	1.78	7	3
	1,450	584	8	2.5	5	7½

TABLE 8.  
COMPARISON OF RESULTS WITH M. L. D. AND WITH L+ DOSE AS STANDARDS.

Days Interval	Result with M. L. D.	Result with L+ Dose	Difference
2	10.5%	20%	+0.5%
4	16	14	-2.0
7	5	6	+1.0

to justify confidence that the curves determined by the two standards are practically the same.

The conclusions that may be drawn from the foregoing are:

1. In passive immunization with diphtheria antitoxin, the degree of protection afforded is always in excess of the protection as calculated from the amount of antitoxin in the blood.
2. Free antitoxin disappears from the blood before immunity is wholly lost by the organism.
3. Diphtheria antitoxin is eliminated by the kidneys, though to but a minor degree.
4. The duration of the protection conferred is practically independent of the quantity of antitoxin administered and of the age of the animal.

The writer wishes to express his thanks to Professor Edwin O. Jordan for advice and suggestions during the course of this study.

IS THE PRESENT METHOD OF STANDARDIZING ANTIDIPHThERIC SERUM ACCORDING TO ANTITOXIN UNITS THERAPEUTICALLY ACCURATE?\*

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CRUVEILHIER<sup>1</sup> quotes Roux, Marfan, Martin, and Momont, as finding that the dose of antidiphtheric serum most efficacious therapeutically is not always the one which contains the greatest number of antitoxic units. The experiences of these authors, Cruveilhier says, seem to indicate that the serum contains, besides the antitoxin, other important preventive and curative substances. In the standardization of antidiphtheric serum as at present practiced, namely, according to antitoxic units, these protective substances are entirely ignored. Cruveilhier to test this point, carried out a series of experiments with guinea-pigs, infected with diphtheria bacilli. He compared sera of different antitoxic strength from several horses as to their value preventively and curatively. In his preventive experiments he injected subcutaneously a quantity of serum proportional to the weight of the animal; and, 24 hours later, inoculated subcutaneously a fatal dose of diphtheria culture. He reported the following results: In four out of seven experiments, with culture No. 261, the animals which received 1/250,000 of 1 c.c., per gram weight, of a 200-unit serum resisted; while those which received the same volume quantity of a 500-unit serum died. In only one instance did the 500-unit serum prove superior, per volume quantity, to the 200-unit serum. Twice the results obtained per volume quantity for both sera were the same. On comparing a 50-unit with one of 500 units, the 50-unit serum proved superior to the 500-unit one, per volume quantity.

With two other cultures, designated "c.c." and "x," he compared a 200-unit serum with a 500-unit one, and obtained practically the

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<sup>1</sup> *Ann. de l'Inst. Past.*, 1904, 18, p. 249.

same results, the 200-unit serum proving more efficacious per volume quantity.

Again, using culture No. 261, he compared a 300-unit serum with one of 500 units, from different bleedings of the same horse, and found them equal per volume in protection.

In his curative experiments, using the same cultures as in the preventive, he injected guinea-pigs subcutaneously with that amount of diphtheria culture which killed the control animal in from 36-48 hours. The animals were divided into two lots. At intervals of two hours, from the second to the 16th hour after inoculation, each animal of one lot received subcutaneously 0.1 c.c. of the serum of the lesser unit content, while each animal of the second lot received 0.1 c.c. of the serum of a greater unit content. In his curative experiments the sera of the lesser unit content were more efficacious than those of the greater.

These results apparently showed that it was the quantity of serum, rather than the number of antitoxic units, which was of therapeutic value.

Cruveilhier drew the following conclusions:

“Que l'effet curatif d'un sérum ne dépend pas exclusivement de sa teneur en unités antitoxiques.

“Que le titrage de l'antitoxine, tel qu'on le pratique habituellement, ne suffit pas à rendre un compte exact de l'efficacité d'un sérum. Que celle-ci est plus exactement appréciée parce que nous avons appelé la mesure du pouvoir thérapeutique.”

In view of the large amount of work which has been done on the titration of diphtheria antitoxin, the results of Cruveilhier cannot but be considered extraordinary.

We have carefully investigated this subject and have obtained results diametrically opposed to those of Cruveilhier and the authors he cites.

In our experiments we compared, both preventively and curatively, native sera and antitoxic globulin solutions (Gibson<sup>1</sup>) of the following unit content: A serum of 43 units compared with an antitoxic globulin solution of 1,700 units; a serum of 43 units with an antitoxic globulin solution of 1,450 units; a serum of 200 units with one of 1,000 units;

<sup>1</sup> *Jour. Biol. Chem.*, 1926, 1, p. 161.



a serum of 600 units with one of 1,300 units; a serum of 600 units with one of 335 units, also with one of 200 units. The last three sera were obtained from the same horse during the course of immunization.

The animals infected were active, healthy normal guinea-pigs weighing between 240 and 260 grams.

The diphtheria bacilli were from the three following strains: Culture No. 1, a moderate toxin producer; culture No. 2, a weak toxin producer. Both of these were freshly isolated from the throats of diphtheria patients at the Department of Health Hospital. Culture No. 8 (Park and Williams), is a strong toxin producer, which has been stock culture in this laboratory for 12 years.

In order to avoid misunderstanding, it should be stated that in the first seven preventive, and the first six curative experiments, the cultures in medium-sized tubes were grown on slant agar for 24 hours in the incubator. Of these,  $\frac{1}{5}$  of a culture was fatal to 250-gram guinea-pigs in 26 to 33 hours. Beginning with experiment 8 preventive, and with experiment 7 curative, the cultures were grown in *large* uniform test-tubes on slant agar. The fatal dose, thereupon, became  $\frac{2}{5}$  culture.

#### PREVENTIVE EXPERIMENTS.

Following the technique of Cruveilhier, a serum<sup>1</sup> of 43 units per c.c. was compared with an antitoxic solution globulin<sup>2</sup> of 1,700 units per c.c.

Four guinea-pigs were injected subcutaneously with 1/150,000 1/200,000, 1/250,000, 1/300,000 of a c.c. per gram weight, of the 43-unit serum, respectively. A parallel set of four guinea-pigs received the same volume per gram weight of the 1,700 unit antitoxic globulin solution; 24 hours later each animal in both series, as well as a control guinea-pig, received subcutaneously a fatal dose of culture No. 8. The control animal died in 24½ hours. The animals which received the 43-unit serum died in 25–28 hours, while those which received the antitoxic globulin solution remained normal. In the above experiment, the 1/150,000 c.c. per gram weight of the 43-unit serum contained 1/25 of a unit; while the same volume of the antitoxic globulin solution contained 2¾ units.

<sup>1</sup> Obtained from Horse 308. This animal had received increasing amounts of toxin since March 6, 1906. The most potent antitoxic value was 250 units on May 3, 1906. Although receiving increasing amounts of toxin every eighth day, the potency dropped steadily, and on July 7, 1906, seven days after the last injection of toxin (1,000 c.c. with a M. L. D. of 0.003), the antitoxic value was about 50 units. At the time of this experiment the serum tested 43 units per c.c.

<sup>2</sup> Prepared from 23 liters of citrated plasma (potency 700 units per c.c.) obtained from four bleedings of Horse 1.

In the second experiment, on account of the great difference in antitoxic unit content, the dilutions were made according to the number of units present.

Four guinea-pigs received  $1/6,250$ ,  $1/12,500$ ,  $1/25,000$ , and  $1/50,000$  c.c. of the 43-unit serum, per gram weight, respectively. Two guinea-pigs received  $1/425,000$  and  $1/850,000$  c.c. per gram weight, respectively, of the 1,700-unit antitoxic globulin solution. Twenty-four hours later, all the animals, as well as a control, received a fatal dose of culture No. 8. Control animal died in 33-36 hours. The animal which received the  $1/50,000$  c.c. of the 43-unit serum showed marked induration and lost  $\frac{1}{3}$  of its weight. The two which received the  $1/425,000$  and  $1/850,000$  c.c. per gram weight of the antitoxic globulin solution remained normal.

In the third experiment made in conjunction with the second, a serum<sup>1</sup> of 200 units was compared with one<sup>2</sup> of 1,000 units.

Three guinea-pigs received  $1/50,000$ ,  $1/100,000$ , and  $1/200,000$  c.c. of the 200-unit serum, per gram weight, respectively. Three others received  $1/250,000$ ,  $1/500,000$ , and  $1/1,000,000$  c.c. of the 1,000-unit serum, per gram weight, respectively. Twenty-four hours later all the animals, as well as a control, received a fatal dose of culture No. 8. Control animal died in 33-36 hours. The animal which received  $1/200,000$  c.c. of the 200-unit serum and the one receiving  $1/1,000,000$  of the 1,000-unit serum showed marked induration and great loss of weight.

In the fourth experiment, four guinea-pigs received  $1/25,000$ ,  $1/37,500$ ,  $1/50,000$ , and  $1/75,000$  c.c. of the 43-unit serum, per gram weight, respectively. Four others received  $1/1,000,000$ ,  $1/1,500,000$ ,  $1/2,000,000$ , and  $1/3,000,000$  c.c. of the 1,700-unit antitoxic globulin solution, per gram weight, respectively. Twenty-four hours later all the animals as well as a control received a fatal dose of culture No. 8. Control died in 28 hours. The animal which received the  $1/37,500$  c.c. of the 43-unit serum was protected; the  $1/50,000$  c.c. failed to

<sup>1</sup> Obtained from Horse 307. This animal had received increasing amounts of toxin since February 14, 1906. The most potent antitoxic value was 350 units on May 3, 1906. Two months later, on July 5, 1906, seven days after the last injection of toxin (850 c.c. with an M. L. D. of 0.003), the antitoxic value was 225 units. At the time of this experiment the serum tested 200 units per c.c.

<sup>2</sup> Obtained from Horse 305. This animal had received increasing amounts of toxin since February 14, 1906. The most potent antitoxic value was 1,250 units on April 17, 1906. On June 12, 1906, seven days after last injection of toxin (500 c.c. with an M. L. D. of 0.002) the antitoxic value was 1,100 units. At the time of this experiment the serum tested 1,000 units per c.c.

protect. The animal which received the  $1/1,500,000$  c.c. of the 1,700-unit antitoxic globulin solution was protected; the  $1/2,000,000$  c.c. failed to protect.

In the fifth experiment, four guinea-pigs received  $1/100,000$ ,  $1/150,000$ ,  $1/200,000$ , and  $1/250,000$  c.c. of the 200-unit serum, per gram weight, respectively. Four others received  $1/500,000$ ,  $1/750,000$ ,  $1/1,000,000$ , and  $1/1,250,000$  c.c. of the 1,000-unit serum, per gram weight, respectively. Twenty-four hours later all the animals, as well as a control, received a fatal dose of culture No. 8. Control animal died in 28 hours. The animal which received the  $1/150,000$  c.c. of the 200-unit serum was protected; the  $1/200,000$  c.c. failed to protect. The animal which received the  $1/750,000$  c.c. of the 1,000-unit serum was protected; the  $1/1,000,000$  c.c. failed to protect.

Thus far our results were not in accord with those of Cruveilhier. In our experiments the therapeutic value of the serum appeared to be measured by the antitoxic unit content, rather than, as his experiments indicated, by the quantity of serum.

TABLE 1.  
PREVENTIVE EXPERIMENTS 1 TO 5.

EXPERIMENT NO. 1 WITH CULTURE NO. 8				EXPERIMENT NO. 3 WITH CULTURE NO. 8			
Fraction of c.c. per Gram Weight	Serum of Horse 308 43 Units per c.c.	Fraction of c.c. per Gram Weight	Antitoxic Glob. Sol. 1,700 Units per c.c.	Fraction of c.c. per Gram Weight	Serum of Horse 307 200 Units per c.c.	Fraction of c.c. per Gram Weight	Serum of Horse 305 1,000 Units per c.c.
$1/150,000$	died	$1/150,000$	lived	$1/50,000$	lived	$1/250,000$	lived
$1/200,000$	died	$1/200,000$	lived	$1/100,000$	lived	$1/500,000$	lived
$1/250,000$	died	$1/250,000$	lived	$1/200,000$	lived	$1/1,000,000$	lived
$1/300,000$	died	$1/300,000$	lived				
Control died in $24\frac{1}{2}$ hrs.				Control died in 33-36 hrs.			
EXPERIMENT NO. 2 WITH CULTURE NO. 8				EXPERIMENT NO. 5 WITH CULTURE NO. 8			
$1/6,250$	lived	$1/425,000$	lived	$1/100,000$	lived	$1/500,000$	lived
$1/12,500$	lived	$1/850,000$	lived	$1/150,000$	lived	$1/750,000$	lived
$1/25,000$	lived			$1/200,000$	died	$1/1,000,000$	died
$1/50,000$	lived			$1/250,000$	died	$1/1,250,000$	died
Control died in 33-36 hrs.				Control died in 28 hrs.			
EXPERIMENT NO. 4 WITH CULTURE NO. 8							
$1/25,000$	lived	$1/1,000,000$	lived				
$1/37,500$	lived	$1/1,500,000$	lived				
$1/50,000$	died	$1/2,000,000$	died				
$1/75,000$	died	$1/3,000,000$	died				
Control died in 28 hrs.							

In experiment 6, the 43-unit serum was compared with an antitoxic globulin solution<sup>1</sup> of 1,450 units. Four guinea-pigs received  $\frac{3}{4}$ ,  $\frac{1}{2}$ ,  $\frac{1}{4}$ , and  $\frac{1}{8}$  unit of the 43-unit serum, respectively. A parallel set of four guinea-pigs received the same fractions of a unit of the 1,450-unit antitoxic globulin solution. Twenty-four hours later all the animals as well as a control received a fatal dose of culture No. 1. Control animal died in 26 hours. The  $\frac{3}{4}$  unit of both the serum and the antitoxic globulin solution protected, while all the animals which received the lower fractions died.

Experiment 7 was identical with the preceding, except that culture No. 2 was used in place of culture No. 1. The results were: Control animal died in 25 hours. The animal which received the  $\frac{1}{2}$  unit of the 43-unit serum became greatly emaciated and barely survived. The  $\frac{3}{4}$  unit of the same serum failed to protect. In all probability this difference was due to an idiosyncrasy of one of the animals. The  $\frac{3}{4}$  unit of the 1,450-unit antitoxic globulin solution protected.

Experiments 6 and 7 were repeated in experiments 8 and 9. The antitoxic values injected were 1,  $\frac{3}{4}$ ,  $\frac{1}{2}$ ,  $\frac{1}{4}$ ,  $\frac{1}{8}$ , and  $\frac{1}{16}$  unit. Using culture No. 1 for experiment No. 8, the results were: Control animal died in 26 hours. The  $\frac{3}{4}$  unit of both the serum and antitoxin globulin solution protected.

In experiment 9, using culture No. 2, in place of culture No. 1, otherwise parallel with experiment 8, the results were: The control animal died in 23½ hours. The one unit of both the serum and the antitoxic globulin solution protected.

In experiment 10, using culture No. 1,<sup>2</sup> a serum<sup>2</sup> of 600 units was compared with one<sup>3</sup> of 1,300 units. The same antitoxic values were administered as in experiments 8 and 9. The results were: Control animal died in 23 hours. The one unit of both sera protected.

In experiment 11, using culture No. 2, in place of culture No.

<sup>1</sup> Prepared from 22 liters of citrated plasma (potency 600 units per c.c.) obtained from two bleedings each of Horses J and L.

<sup>2</sup> Obtained from Horse N. This animal had received increasing amounts of toxin since October 5, 1906. The most potent antitoxic value was 700 units on February 26, 1907, seven days after the last injection of toxin. Rebleeding one week later, the potency had dropped to 600 units. This was its value also at the time of our experiments, two weeks later.

<sup>3</sup> Obtained from Horse 306. This animal had received increasing amounts of toxin since February 14, 1906. The most potent antitoxic value was 1,450 units on May 18, 1906, six days after last injection of toxin (550 c.c. with an M. L. D. of 0.002). At the time of these experiments the serum tested 1,300 units per c.c.

1, otherwise parallel with experiment 10, the results were: Control animal died in 33-36 hours. The  $\frac{1}{4}$  unit of both sera barely protected.

In experiment 12, using culture No. 8, in place of No. 2, otherwise parallel with experiments 10 and 11, the results were: Control animal died in 28 hours. The  $\frac{3}{4}$  unit of the 600-unit serum protected; the  $\frac{1}{2}$  unit of the 1,300-unit serum barely protected.

In the two following experiments (13 and 14) comparisons were made of the three sera<sup>1</sup> of different unit content obtained from the same horse during the course of immunization.

In experiment 13, using culture No. 1, five guinea-pigs received 1,  $\frac{3}{4}$ ,  $\frac{1}{2}$ ,  $\frac{1}{4}$ , and  $\frac{1}{8}$  unit of a 600-unit serum respectively, five others received the same antitoxic unit values of a 335-unit serum; five more received the same values of a 200-unit serum. Twenty-four hours later all the animals, as well as a control, received a fatal dose of culture No. 1. The results were: Control animal died in 28 hours. The  $\frac{1}{2}$  unit of each of the three sera protected.

In experiment 14, using culture No. 8 in place of culture No. 1, otherwise parallel with experiment No. 13, the results were: Control animal died in 30 hours. The  $\frac{1}{2}$  unit of each of the three sera protected.

To summarize: In experiments 1, 2, 4, 6, 7, 8, and 9, we compared a 43-unit native serum with a 1,700-unit and a 1,450-unit antitoxin globulin solution. The results, in each case, are seen to depend on the antitoxic unit content of the serum and not on its volume. The same is true of experiments 3 and 5, in which a 200-unit serum was compared with one of 1,000 units, and of experiments 10, 11, and 12, in which a fresh serum (two weeks old) of 600 units was compared with one (10 months old) of 1,300 units. Finally the same results are seen in experiments 13 and 14, in which we compared three bleedings from the same horse as follows: One bleeding originally contained 625 units per c.c. (this was the maximum value attained by the animal). The second contained 335 units per c.c., and the third contained 200 units per c.c.

<sup>1</sup> Obtained from Horse 322. This animal had received increasing amounts of toxin since March 7, 1907. The most potent antitoxic value was 625 units, on April 30, 1907. Tested at the time of these experiments the serum contained 600 units per c.c. After the fourth bleeding, which was on July 7, 1907, the antitoxic value had dropped to 340 units; tested at the time of these experiments the serum contained 335 units per c.c. After three further bleedings, the antitoxic value dropped to 200 units on June 28, 1907. This was the potency also at the time of these experiments.

TABLE 2.  
PREVENTIVE EXPERIMENTS 6 TO 14.

AMOUNT INJECTED IN UNITS	EXPERIMENT NO. 6 WITH CULTURE NO. 1		EXPERIMENT NO. 10 WITH CULTURE NO. 1		EXPERIMENT NO. 13 WITH CULTURE NO. 1		
	Serum of Horse 308 43 Units per c.c.	Antitoxic Glob. Sol. 1,450 Units per c.c.	Serum of Horse N 600 Units per c.c.	Serum of Horse 306 1,300 Units per c.c.	Serum of Horse 322 600 Units per c.c.	Serum of Horse 322 335 Units per c.c.	Serum of Horse 322 200 Units per c.c.
1 unit			lived	lived	lived	lived	lived
$\frac{3}{4}$ "	lived	lived	died	died	lived	lived	lived
$\frac{1}{2}$ "	died	died	died	died	lived	lived	lived
$\frac{1}{4}$ "	died	died	died	died	died	died	died
$\frac{1}{8}$ "	died	died	died	died	died	died	died
$\frac{1}{16}$ "			died	died			
	Control died in 26 hrs.		Control died in 23 hrs.		Control died in 28 hrs.		
AMOUNT INJECTED IN UNITS	EXPERIMENT NO. 7 WITH CULTURE NO. 2		EXPERIMENT NO. 11 WITH CULTURE NO. 2		EXPERIMENT NO. 14 WITH CULTURE NO. 8		
1 unit			lived	lived	lived	lived	lived
$\frac{3}{4}$ "	died	lived	lived	lived	lived	lived	lived
$\frac{1}{2}$ "	lived	died	lived	lived	lived	lived	lived
$\frac{1}{4}$ "	died	died	lived	lived	died	died	died
$\frac{1}{8}$ "	died	died	died	died	died	died	died
$\frac{1}{16}$ "			died	died			
	Control died in 25 hrs.		Control died in 33-36 hrs.		Control died in 30 hrs.		
AMOUNT INJECTED IN UNITS	EXPERIMENT NO. 8 WITH CULTURE NO. 1		EXPERIMENT NO. 12 WITH CULTURE NO. 8				
1 unit			lived	lived			
$\frac{3}{4}$ "	lived	lived	lived	lived			
$\frac{1}{2}$ "	died	died	died	lived			
$\frac{1}{4}$ "	died	died	died	died			
$\frac{1}{8}$ "	died	died	died	died			
$\frac{1}{16}$ "	died	died	died	died			
	Control died in 26 hrs.		Control died in 28 hrs.				
AMOUNT INJECTED IN UNITS	EXPERIMENT NO. 9 WITH CULTURE NO. 2						
1 unit							
$\frac{3}{4}$ "	lived	lived					
$\frac{1}{2}$ "	died	died					
$\frac{1}{4}$ "	died	died					
$\frac{1}{8}$ "	died	died					
$\frac{1}{16}$ "	died	died					
	Control died in 23½ hrs.						

## CURATIVE EXPERIMENTS.

*Experiment 1.*—Following Cruveilhier's technique, we infected the animals with culture No. 8, and divided them into two lots. At intervals of two hours, from the second to the tenth hour, each animal of the first lot received subcutaneously 25 units of the 43-unit serum; and each animal of the second lot, 25 units of the 1,700-unit

antitoxic globulin solution. The results of both lots were uniform. Control animal died in 25 hours. The animals which received the curative dose after 2 and 4 hours lived; while those receiving the curative dose after 6, 8, and 10 hours, died.

*Experiment 2.*—The animals were infected with culture No. 1, and divided into two lots. At intervals of two hours, from the second to the eighth hour, each animal of the first lot received subcutaneously 25 units of the 43-unit serum; and each animal of the second lot, 25 units of the 1,450-unit antitoxic globulin solution. The results of both lots were uniform. Control animal died in 26 hours. The animals which received the curative dose after 2, 4, and 6 hours lived; while those receiving the curative dose after 8 hours, died.

*Experiment 3.*—The animals were infected with culture No. 2, in place of culture No. 1; otherwise parallel with experiment 2. The results of both lots were again uniform. The control animal died in 26 hours. The animals which received the curative dose after 2 and 4 hours lived; after 6 and 8 hours, died.

*Experiment 4.*—Repeating experiment 2, in duplicate, the result of both lots were again uniform. The control animal died in 21½ hours. The animals which received the curative dose after 2 and 4 hours lived; after 6 and 8 hours, died.

*Experiment 5.*—Repeating experiment 3, in duplicate, the results were again uniform. The control animal died in 28 hours. The animals which received the curative dose after 2, 4, and 6 hours, lived; after 8 hours, died.

*Experiment 6.*—The animals were injected with culture No. 1 and divided in two lots. At intervals of two hours, from the fourth to the eighth hour, each animal of the first lot received subcutaneously 25 units of the 600-unit serum. The second lot received 25 units of the 1,300-unit serum. The results of both lots were uniform. The control animal died in 27 hours. The animals which received the curative dose after 4 and 6 hours, lived; after 8 hours, died.

*Experiment 7.*—The animals were infected with culture No. 2 in place of culture No. 1, otherwise parallel with experiment No. 6. The results of both lots were uniform. The control animal died in 28 hours. The animals which received the curative dose after 4 and 6 hours lived; after 8 hours, died.

TABLE 3.  
CURATIVE EXPERIMENTS 1 TO 11.

HOURS ELAPSED AFTER INJECTION OF CULTURE	EXPERIMENT 1 WITH CULTURE 8		EXPERIMENT 2 WITH CULTURE 1		EXPERIMENT 6 WITH CULTURE 1		EXPERIMENT 9 WITH CULTURE 1			
	Serum of Horse 308 43 Units per c.c. (25 Units Injected)	Antitoxic Glob. Sol. 1,700 Units per c.c. (25 Units Injected)	Serum of Horse 308 43 Units per c.c. (25 Units Injected)	Antitoxic Glob. Sol. 1,450 Units per c.c. (25 Units Injected)	Serum of Horse N 600 Units per c.c. (25 Units Injected)	Serum of Horse 306 1,300 Units per c.c. (25 Units Injected)	Serum of Horse 322 600 Units per c.c. (25 Units Injected)	Serum of Horse 322 335 Units per c.c. (25 Units Injected)	Serum of Horse 322 200 Units per c.c. (25 Units Injected)	
2 hours	lived	lived	lived	lived	lived	lived	lived	lived	lived	
4 "	died	died	lived	lived	lived	lived	died	died	died	
6 "	died	died	died	died	died	died	died	died	died	
8 "	died	died	died	died	died	died	died	died	died	
10 "	died	died	Control died in 25 hrs.		Control died in 26 hrs.		Control died in 22 hrs.			
2 hours	EXPERIMENT 3 WITH CULTURE 2		EXPERIMENT 7 WITH CULTURE 2		EXPERIMENT 10 WITH CULTURE 8					
	lived	lived	lived	lived	lived	lived				
4 "	died	died	died	died	died	died				
6 "	died	died	died	died	died	died				
8 "	died	died	died	died	died	died				
		Control died in 26 hrs.		Control died in 28 hrs.		Control died in 24½ hrs.				
2 hours	EXPERIMENT 4 WITH CULTURE 1 (IN DUPLICATE)		EXPERIMENT 8 WITH CULTURE 8		EXPERIMENT 11 WITH CULTURE 2					
	2 lived	2 lived	2 lived	2 lived	lived	lived				
4 "	2 lived	2 lived	2 lived	2 lived	lived	lived				
6 "	2 died	2 died	2 died	2 died	died	died				
8 "	2 died	2 died	2 died	2 died	died	died				
		Control died in 21½ hrs.		Control died in 27 hrs.		Control died in 27 hrs.				
2 hours	EXPERIMENT 5 WITH CULTURE 2 (IN DUPLICATE)		EXPERIMENT 10 WITH CULTURE 8		EXPERIMENT 11 WITH CULTURE 2					
	2 lived	2 lived	2 lived	2 lived	lived	lived				
4 "	2 lived	2 lived	2 lived	2 lived	lived	lived				
6 "	2 lived	2 lived	2 lived	2 lived	died	died				
8 "	2 died	2 died	2 died	2 died	died	died				
		Control died in 28 hrs.		Control died in 28 hrs.		Control died in 27 hrs.				



*Experiment 8.*—The animals were infected with culture No. 8, in place of culture No. 2, otherwise parallel with experiments 6 and 7. The results of both lots were uniform. The control died in 27 hours. The animals which received the curative dose after 4 and 6 hours lived; after 8 hours, died.

*Experiment 9.*—The animals were infected with culture No. 1 and divided into three lots. At intervals of two hours, from the fourth to the eighth hour, each animal of the first lot received subcutaneously 25 units of the 600-unit serum, the second lot, 25 units of the 335-unit serum, and the third lot received the same number of units of the 200-unit serum. These three sera were obtained from the same horse during the course of immunization. The results of the three lots were uniform. The control died in 22 hours. The animals which received the curative dose after 4 hours lived; after 6 and 8 hours, died.

*Experiment 10.*—The animals were infected with culture No. 8 in place of culture No. 1, otherwise parallel with experiment 9. The results of the three lots were uniform. The control animal died in 24½ hours. Those which received the curative dose after 4 hours lived; after 6 and 8 hours, died.

*Experiment 11.*—The animals were infected with culture No. 2, in place of culture No. 1; otherwise parallel with experiments 9 and 10. The results of the three lots were uniform. The control died in 27 hours. Those which received the curative dose after 4 and 6 hours lived; after 8 hours, died.

#### CONCLUSIONS.

In view of the results obtained in the comparison of the antitoxic content of sera, both preventively and curatively, it is obvious that in diphtheria in the guinea-pig, the therapeutic value of antidiphtheric sera depends on the number of antitoxic units present. There is no reason for believing that conditions in man are different.

According to our experiments antidiphtheric serum contains no protective substances, aside from the antitoxin, which play an important rôle therapeutically.

The present method of standardizing antidiphtheric serum accurately measures its therapeutic value.

# THE RELATIVE THERAPEUTIC VALUE OF ANTITOXIC GLOBULIN SOLUTION AND THE WHOLE SERUM FROM WHICH IT WAS DERIVED.\*

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THE process of concentrating diphtheria antitoxin, devised in this laboratory by Gibson,<sup>1</sup> has been adopted by a large number of American manufacturers, and has resulted in the extensive employment of the product in this country. Careful clinical trials have convinced us that this antitoxic globulin preparation possesses all the therapeutic properties of the native serum.

Cruveilhier<sup>2</sup> has recently stated that the customary method of standardizing antidiphtheric serum is not comprehensive enough, in that it leaves out of account therapeutic serum-constituents other than the antitoxin. While we believe that the work of Steinhardt and Banzhaf in the preceding paper<sup>3</sup> has effectually disposed of this objection, it seemed advisable to study the question in connection with the Gibson process of concentration, and with the fractional precipitation of the antiserum according to the method of Banzhaf and Gibson.<sup>4</sup>

Our general method of procedure was similar to that outlined in the preceding paper. In this instance, we mixed antitoxic citrated plasma from several horses and then tested this mixture before and after eliminating the non-antitoxic proteins. We also compared the different globulin fractions of the plasma.

In these experiments, the following mixtures of citrated plasma were used:

*Mixture 1.*—Fifty liters of citrated plasma were obtained from five horses as follows:

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<sup>1</sup> *Jour. Biol. Chem.*, 1, p. 161.

<sup>3</sup> *Jour. Infect. Dis.*, 1908, 5, p. 203.

<sup>2</sup> *Ann. de l'Inst. Past.*, 1904, 18, p. 240.

<sup>4</sup> *Jour. Biol. Chem.*, 3, p. 253.

1,000 c.c.	from horse 262,	part of one bleeding.
3,600 c.c.	" "	305, " " two bleedings.
5,400 c.c.	" "	306, " " three bleedings.
1,500 c.c.	" "	310, " " one bleeding.
38,500 c.c.	" "	311, full six bleedings.

The potency of this mixture, at the time of these experiments, was 700 units per c. c.

*Mixture 2.*—Forty liters of citrated plasma were obtained from three horses as follows:

8,000 c.c.	from horse 288,	full bleeding.
6,100 c.c.	" "	305, full bleeding.
25,900 c.c.	" "	299, full four bleedings.

The original potency of this mixture was 475 units per c.c. At the time of these experiments, however, the potency had dropped to 450 units per c.c.

(Mixture 1.) The 50 liters were refined and concentrated by Gibson's method, to 16 liters of antitoxic globulin solution containing 1,650 units per c.c.

Gibson's method briefly is as follows: The diluted citrated plasma is precipitated with an equal amount of saturated ammonium sulphate solution and the antitoxic proteins are separated by extracting the precipitate with saturated sodium chloride solution. The soluble antitoxic proteins are then reprecipitated from the saturated sodium chloride solution with acetic acid. This filtered precipitate is then partially dried between filter papers and dialyzed in parchment in running water.

(Mixture 2.) The 40 liters were divided into two lots. One lot of 20 liters was diluted with an equal amount of water and refined and concentrated by Gibson's method. The final product, designated 77A, amounted to 5,200 c.c., each c.c. containing 1,450 units per c.c. At the time of these experiments, however, the potency had dropped to 1,375 units per c.c.

The second lot of 20 liters was diluted with an equal amount of water and the antitoxic globulins obtained by three fractional precipitations at concentrations corresponding to 3.3 c.c., 3.3-3.8 c.c., and 3.8-5.0 c.c. of saturated ammonium sulphate in 10 c.c.<sup>1</sup>

<sup>1</sup> There exists at the present time considerable confusion in comprehending the methods and basic principles of ammonium sulphate fractional precipitation of proteins. The nomenclature which we have employed and which designates the number of c.c. of saturated ammonium sulphate solution in 10 c.c.

The soluble antitoxic proteins in saturated sodium chloride solution were prepared in the usual way. The antitoxic globulin solution from the first fraction, designated 77B, amounted to 1,440 c.c., each c.c. containing 1,150 units per c.c. At the time of these experiments, however, the potency had dropped to 1,025 units per c.c. The second fraction, designated 77C, amounted to 1,400 c.c., each c.c. containing 1,350 units per c.c. At the time of these experiments, however, its potency had dropped to 1,175 units per c.c. The third fraction, designated 77D, amounted to 2,050 c.c., each c.c. containing 1,750 units per c.c. At the time of these experiments, however, the potency had dropped to 1,550 units per c.c.

The animals used in these experiments were active, healthy, normal guinea-pigs weighing between 250–260 grams.

The three diphtheria strains were the same as in the preceding paper.

#### PREVENTIVE EXPERIMENTS.

##### *Mixture 1.*

A uniform sample of the mixture of citrated plasma containing 700 units per c.c., was compared with the finished product of antitoxic globulin solution containing 1,650 units per c.c.

*Experiment 1.*—Six guinea-pigs were inoculated subcutaneously with 1,  $\frac{3}{4}$ ,  $\frac{1}{2}$ ,  $\frac{1}{4}$ ,  $\frac{1}{8}$ , and  $\frac{1}{16}$ -unit of the 700-unit citrated plasma, respectively; a parallel set of six guinea-pigs received the same antitoxic unit values of the 1,650 antitoxic globulin solution. Twenty-four hours later, all the animals, as well as a control guinea-pig, received a fatal dose of culture No. 1.<sup>1</sup> The control animal died in 27 hours. The  $\frac{3}{4}$ -unit of both the citrated plasma and the antitoxic globulin solution protected.

*Experiment 2.*—In this, culture No. 2<sup>1</sup> was used in place of culture No. 1; otherwise it was parallel with the first experiment. The results were: The control animal died between 33 and 36 hours.

of the *precipitated* mixture has been used by some authorities; it avoids the confusion developed by the use of such terms as "per cent  $(\text{NH}_4)_2\text{SO}_4$  solution," "per cent saturation  $(\text{NH}_4)_2\text{SO}_4$ ," "per cent of saturated  $(\text{NH}_4)_2\text{SO}_4$  solution," and "per cent saturation  $(\text{NH}_4)_2\text{SO}_4$  solution," and it seems the simplest and best *practical* expression of degrees of saturation yet suggested. We advise that this method be employed in future papers on fractional precipitation.

<sup>1</sup> Culture Nos. 1 and 2: No. 1, a moderate toxin producer; No. 2, a weak toxin producer. Both of these were freshly isolated from the throats of diphtheria patients at the Department of Health Hospital.

The  $\frac{1}{4}$ -unit of both the citrated plasma and the antitoxic globulin solution protected.

*Experiment 3.*—In this, culture No. 8<sup>1</sup> was used in place of culture No. 2; otherwise it was parallel with the first and second experiments. The results were: The control animal died in 28 hours. The  $\frac{1}{2}$ -unit of both the citrated plasma and the antitoxic globulin solution protected.

TABLE 1.  
PREVENTIVE EXPERIMENTS.

AMOUNT INJECTED IN UNITS	EXPERIMENT 1 WITH CULTURE NO. 1		EXPERIMENT 4 WITH CULTURE NO. 1				
	Sample of Plasma 700 Units per c.c.	Concen- trated Anti- toxic Glob. Sol. 1,650 Units per c.c.	Sample of Plasma 450 Units per c.c.	Concen- trated Anti- toxic Glob. Sol. 77A 1,375 Units per c.c.	First Fract. Antitoxic Glob. Sol. 77B 1,025 Units per c.c.	Second Fract. Anti- toxic Glob. Sol. 77C 1,175 Units per c.c.	Third Fract. Antitoxic Glob. Sol. 77D 1,550 Units per c.c.
1 unit	lived	lived	lived	lived	lived	lived	lived
$\frac{1}{2}$ "	lived	lived	lived	lived	lived	lived	lived
$\frac{1}{4}$ "	died	died	lived	lived	lived	lived	lived
$\frac{1}{8}$ "	died	died	died	died	died	died	died
$\frac{1}{16}$ "	died	died	died	died	died	died	died
	Control died in 27 hrs.		Controls died in 28½ and 29 hrs.				
	EXPERIMENT 2 WITH CULTURE NO. 2		EXPERIMENT 5 WITH CULTURE NO. 2				
1 unit	lived	lived	lived	lived	lived	lived	lived
$\frac{1}{2}$ "	lived	lived	lived	lived	lived	lived	lived
$\frac{1}{4}$ "	lived	lived	lived	lived	lived	lived	lived
$\frac{1}{8}$ "	lived	lived	died	died	died	died	died
$\frac{1}{16}$ "	died	died	died	died	died	died	died
	Control died in 33-36 hrs.		Controls died in 28½ hrs.				
	EXPERIMENT 3 WITH CULTURE NO. 8		EXPERIMENT 6 WITH CULTURE NO. 8				
1 unit	lived	lived	lived	lived	lived	lived	lived
$\frac{1}{2}$ "	lived	lived	lived	lived	lived	lived	lived
$\frac{1}{4}$ "	lived	lived	lived	lived	lived	lived	lived
$\frac{1}{8}$ "	died	died	lived	lived	lived	lived	lived
$\frac{1}{16}$ "	died	died	died	died	died	died	died
	Control died in 28 hrs.		Controls died in 30-32 hrs.				

### Mixture 2.

In Experiments 4, 5, and 6, we instituted the following comparisons:

a) Between a sample of citrated plasma (Mixture 2) containing

<sup>1</sup> Park and Williams, culture No. 8. This is a strong toxin producer and has been the stock in this laboratory for 12 years.

450 units per c.c., and the finished product of antitoxic globulin solution containing 1,375 units per c.c.

b) Between the same citrated plasma (Mixture 2) and the three fractions of the antitoxic globulins; of these Fraction 1 contained 1,025 units per c.c.; Fraction 2 contained 1,175 units per c.c.; and Fraction 3, 1,550 units per c.c.

*Experiment 4.*—This required 30 guinea-pigs as follows: Six guinea-pigs received 1,  $\frac{3}{4}$ ,  $\frac{1}{2}$ ,  $\frac{1}{4}$ ,  $\frac{1}{8}$ , and  $\frac{1}{16}$ -unit of the 450-unit citrated plasma, respectively; six received the same antitoxic unit values of the 1,375-unit antitoxic globulin solution, 77A; six received the same antitoxic values of the 1,025-unit, first fraction, antitoxic globulin solution; six received the same antitoxic values of the 1,175-unit, second fraction, antitoxic globulin solution; six received the same values of the 1,550-unit, third fraction, antitoxic globulin solution. Twenty-four hours later, all the animals, as well as two control guinea-pigs, received a fatal dose of culture No. 1. The results of the five lots were uniform. The control animals died between 28½ and 29 hours. The ½-unit of the citrated plasma, the antitoxic globulin solution, and the fractions of the antitoxic globulins protected.

*Experiment 5.*—In this, culture No. 2 was used in place of culture No. 1; otherwise it was parallel with experiment 4. The results of the five lots were uniform. The control animals died in 28½ hours. The ½-unit of the citrated plasma, the antitoxic globulin solution, and the fractions of the antitoxic globulins protected.

*Experiment 6.*—In this, culture No. 8 was used in place of culture No. 2; otherwise it was parallel with experiments 4 and 5. The results of the five lots were uniform. The control animals died between 30 and 32 hours. The ¼-unit of the citrated plasma, the antitoxic globulin solution, and the fractions of the antitoxic globulins protected.

#### CURATIVE EXPERIMENTS.

##### *Mixture 1.*

The guinea-pigs were inoculated subcutaneously with a fatal dose of culture No. 1, and divided into two lots. At intervals of two hours from the fourth to the eighth hour, each animal of the first lot received subcutaneously 25 units of the 700-unit citrated plasma;

and each animal of the second lot received 25 units of the 1,650-unit antitoxic globulin solution. The results were: The control animal died in 27 hours. The animals receiving the curative dose after four and six hours, lived; while those receiving the curative dose after eight hours, died.

TABLE 2.  
CURATIVE EXPERIMENTS.

NO. OF HOURS AFTER INJECTION OF CULTURE	EXPERIMENT 1 WITH CULTURE No. 1		EXPERIMENT 4 WITH CULTURE No. 1				
	Sample of Plasma 700 Units per c.c. (25 Units Injected)	Concentrated Antitoxic Glob. Sol. 1,650 Units per c.c. (25 Units Injected)	Sample of Plasma 450 Units per c.c. (25 Units Injected)	Concentrated Antitoxic Glob. Sol. 77A 1,375 Units per c.c. (25 Units Injected)	First Fract. Antitoxic Glob. Sol. 77B 1,025 Units per c.c. (25 Units Injected)	Second Fract. Antitoxic Glob. Sol. 77C 1,175 Units per c.c. (25 Units Injected)	Third Fract. Antitoxic Glob. Sol. 77D 1,550 Units per c.c. (25 Units Injected)
4 hours	lived	lived	lived	lived	lived	lived	lived
6 "	lived	lived	lived	lived	lived	lived	lived
8 "	died	died	died	died	died	died	died
	Control died in 27 hrs.		Controls died in 28 hrs.				
	EXPERIMENT 2 WITH CULTURE No. 2		EXPERIMENT 5 WITH CULTURE No. 2				
	Sample of Plasma 700 Units per c.c. (25 Units Injected)	Concentrated Antitoxic Glob. Sol. 1,650 Units per c.c. (25 Units Injected)	Sample of Plasma 450 Units per c.c. (25 Units Injected)	Concentrated Antitoxic Glob. Sol. 77A 1,375 Units per c.c. (25 Units Injected)	First Fract. Antitoxic Glob. Sol. 77B 1,025 Units per c.c. (25 Units Injected)	Second Fract. Antitoxic Glob. Sol. 77C 1,175 Units per c.c. (25 Units Injected)	Third Fract. Antitoxic Glob. Sol. 77D 1,550 Units per c.c. (25 Units Injected)
4 hours	lived	lived	lived	lived	lived	lived	lived
6 "	lived	lived	lived	lived	lived	lived	lived
8 "	died	died	died	died	died	died	died
	Control died in 26½ hrs.		Controls died in 27-27½ hrs.				
	EXPERIMENT 3 WITH CULTURE No. 8		EXPERIMENT 6 WITH CULTURE No. 8 (IN DUPLICATE)				
	Sample of Plasma 700 Units per c.c. (25 Units Injected)	Concentrated Antitoxic Glob. Sol. 1,650 Units per c.c. (25 Units Injected)	Sample of Plasma 450 Units per c.c. (25 Units Injected)	Concentrated Antitoxic Glob. Sol. 77A 1,375 Units per c.c. (25 Units Injected)	First Fract. Antitoxic Glob. Sol. 77B 1,025 Units per c.c. (25 Units Injected)	Second Fract. Antitoxic Glob. Sol. 77C 1,175 Units per c.c. (25 Units Injected)	Third Fract. Antitoxic Glob. Sol. 77D 1,550 Units per c.c. (25 Units Injected)
4 hours	lived	lived	2 lived	2 lived	2 lived	2 lived	2 lived
6 "	died	died	2 died	2 died	2 died	2 died	2 died
8 "	died	died	2 died	2 died	2 died	2 died	2 died
	Control died in 25½ hrs.		Controls died in 25 hrs.				

*Experiment 2.*—In this, culture No. 2 was used in place of culture No. 1; otherwise it was parallel with the first experiment. The results were: The control animal died in 26½ hours. The animals receiving the curative dose after four and six hours, lived; while those receiving the curative dose after eight hours, died.

*Experiment 3.*—In this, culture No. 8 was used in place of culture No. 2; otherwise it was parallel with experiments 1 and 2. The results were: The control animal died in 25½ hours. The animals receiving the curative dose after four hours, lived; after six and eight hours, died.

*Mixture 2.*

*Experiment 4.*—The guinea-pigs received subcutaneously a fatal dose of culture No. 1, and were divided into five lots. At intervals of two hours from the fourth to the eighth hour, each animal of the first lot received subcutaneously 25 units of the 450-unit citrated plasma; each animal of the second lot 25 units of the 1,375-unit antitoxic globulin solution, 77-A; the third lot 25 units of the 1,025-unit, first fraction, antitoxic globulin solution; the fourth lot 25 units of the 1,175-unit, second fraction, antitoxic globulin solution; the fifth lot 25 units of the 1,550-unit, third fraction, antitoxic globulin solution. The results of the five lots were uniform. The control animals died in 28 hours. All the animals receiving the curative dose after four and six hours, lived; after eight hours, died.

*Experiment 5.*—In this, culture No. 2 was used in place of culture No. 1; otherwise it was parallel with experiment 4. The results of the five lots were again uniform. The control animals died in 27 and 27½ hours. All the animals receiving the curative dose after four and six hours, lived; after eight hours, died.

*Experiment 6.*—In this, which was in duplicate, culture No. 8 was used, in place of culture No. 2; otherwise it was parallel with experiments 4 and 5. The duplicate results of the five lots were again uniform. The control animals died in 25 hours. All the animals receiving the curative dose after four hours, lived; after six and eight hours, died.

In view of the results obtained in these experiments and in those of Steinhardt and Banzhaf in the preceding paper, it is obvious that the therapeutic value of the plasma is not appreciably impaired through the process of eliminating the albumins and other non-antitoxic proteins by the salting out methods employed, and the final dialyzation of the concentrated product.



# STUDIES ON IMMUNITY IN ROCKY MOUNTAIN SPOTTED FEVER.\*

## FIRST COMMUNICATION.†

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In these studies it has been our aim to ascertain with certainty some of the fundamental facts concerning immunity to this disease and to pass as rapidly as possible to the practical aspects of the question, namely, the study of serum prevention, serum therapy, and specific vaccination. Concerning these three important phases of the work, we report preliminary experiments which indicate the scope of our studies, the results of which will be reported more exhaustively in the future.

### DIAGNOSIS OF THE EXPERIMENTAL DISEASE.

Before considering the experiments it seems desirable to point out the essential clinical and anatomical features of experimental spotted fever in order to show that the criteria for the recognition and differentiation of the disease are of a definite and convincing character. While diagnosis is usually easy concerning infections caused by organisms that may be cultivated and that show definite biologic characteristics, or that have a definite morphology, as in the case of certain protozoa, it would appear to be more difficult and liable to error when the organism is unrecognized and uncultivated.

The incubation period is definite and is never absent in animals that were fever free and that suffered no accident at the time of inoculation such as might be caused by puncture of the intestines or some accidental infection. Following intraperitoneal inoculation in the guinea-pig, monkey, or rabbit,<sup>1</sup> two to four or five days elapse before a distinct rise in the temperature occurs. The incubation period is from two to four days longer when the inoculation is subcutaneous. Its length also has a certain relation to the quantity of virus inoculated; when the minimum pathogenic dose is used it is often from one to three days longer than when several multiples of this dose are injected.

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<sup>1</sup> The rabbit has recently been found to be susceptible to the strains now cultivated in laboratory animals.

At the close of the incubation period the temperature rises to a maximum rapidly. Not infrequently it is normal (about 102.5) on one day, and on the next day, 105 or even 106.<sup>1</sup> More often, however, the first day of the febrile period is marked by a temperature of 103 to 104 with a more pronounced rise on the second and following days. The course is that of a continuous fever throughout, as in man. For working purposes it is satisfactory to take the temperature but once a day and it is immaterial whether this is done in the morning or afternoon. Sometimes the maximum temperature is not reached until the third or fourth day of fever. Usually the high point is about 106, although a temperature of 107.2 has been observed.

In fatal cases the duration of the fever is subject to variations, some dying after four or five days and others after eight or ten days of fever. Similarly animals which recover may exhibit fever for from six to ten days. In recovery subsidence of the temperature is usually gradual. Occasionally, however, a drop to normal is noted within 24 hours, and it may be slightly below normal for one or two days. In fatal cases the reduction is sudden, and a drop to 97 or 99 is an unfailing sign of impending death.

The most characteristic external signs of the disease are emaciation, a roseolar eruption, hemorrhages into and gangrene of the external genitalia. The roseolar eruption begins on the third to the fifth day of fever and is best seen on the external genitalia of guinea-pigs with white skins. If such animals are shaved it may also be detected on other parts of the body, especially the thighs, back and face. It is not always recognizable and escapes observation entirely on animals having dark skins. In males the scrotum begins to swell after from two to four days of fever and it is characteristic for the skin at this point to become densely infiltrated with blood; in the event that the animal lives long enough, i. e., in the event of recovery, the hemorrhagic areas become gangrenous and separate, leaving greater or less deformity of the scrotum. These phenomena are not absolutely constant but occur in a high percentage of the males. Apparently the swelling of the scrotum is due chiefly to congestion and edema of the underlying connective tissue, the tunica vaginalis and the epididymis. In females, the vulva becomes swollen and may show the roseolar eruption as stated; hemorrhage and gangrene of the vulva, however, are rather uncommon. Frequently the ears suffer from gangrene to a greater or less extent and it is the impression that this phenomenon now occurs more frequently than when the virus was first cultivated from man. Occasionally both ears fall away to their bases. Less frequently the soles of the feet undergo gangrene. In fatal cases the guinea-pig loses approximately one-third of its weight during the disease.

The most characteristic findings at autopsy, aside from the changes in the genitalia, are a greatly enlarged spleen and enlarged and hemorrhagic lymph glands with extreme congestion of the adjacent areolar tissue. Enlargement of the suprarenal glands is of frequent occurrence and they sometimes contain small hemorrhages.

<sup>1</sup> It has been the rule in taking the temperature of guinea-pigs to insert the thermometer as far as possible into the colon. If this distance is not more than two inches, it is usually because the colon is occluded by feces, and this condition is recorded at such times. In animals of 300 to 400 grams weight it is usually possible to reach a depth of about three inches, and in full grown guinea-pigs the thermometer can be inserted for almost its entire length. (This is the ordinary clinical thermometer.) A large number of observations indicate that the normal temperature of the guinea-pig, as taken in this way, lies between 102.2 and 102.6. A temperature above 103 would seem to be abnormal, although occasional groups of animals are encountered in which the temperatures may vary between 103 and 104.5, without any evident cause. This may persist for several days and, naturally, causes confusion in the recognition of spotted fever.

A valuable point for the corroboration of the diagnosis is the failure to cultivate any microorganism from the blood or organs of animals that have been killed during the course of the fever, or which have died as a natural result of the infection. This statement refers to typical cases, in which the clinical course indicates that there was no mixed infection, and in the case of autopsies it applies to those that are performed immediately or soon after death.

This negative bacteriologic finding has been constant through two years of work and although it is of the greatest importance as a diagnostic point, the clinical course of the disease is so characteristic that cultures are now resorted to only occasionally to prove the purity of the virus used for passage, and in experiments to serve as a check for animals in which there has been some departure from the normal course of the disease or in which the autopsy reveals unusual conditions. If the animals used for an experiment are free from fever when the experiment is begun and if following injection they or their controls exhibit the usual incubation period followed by a typical course of fever and the development of the cutaneous phenomena and if the findings at autopsy are as described above the experiments are sufficiently controlled for working purposes. This method has been found perfectly satisfactory and in two instances was sufficient for the recognition of adventitious epidemics, the presence of which was subsequently verified by bacteriologic methods.

In view of the fact, as stated later, that an attack of spotted fever, however mild, confers strong immunity, an "immunity test" is of value in determining whether animals which recover have or have not suffered from spotted fever. Our experience indicates that any used animal which develops spotted fever after the injection of 20 to 50 multiples of the minimum pathogenic dose of virus could not have had the disease in the first instance.

#### THE VIRUS.

For the major part of the work two strains of the virus have been used, one obtained in the spring of 1906 and the other in 1907. The first is known as the Bradley strain, the second as the Eddy strain. During the summer of 1906 the Bradley strain was grown alternately in monkeys and guinea-pigs and subsequently in guinea-pigs alone. The Eddy strain has been cultivated in guinea-pigs exclusively. The method of cultivation is that of the ordinary passage, the blood of an infected animal being injected intraperitoneally or subcutaneously into a healthy one. The Bradley strain has undergone approximately 125 passages. The two strains show about the same degree of virulence judging from the quantity of virulent blood required to produce infection. On this account they have been used more or less interchangeably, a record being kept of the strain used for each experiment.

It is important to know whether the virus used in these experiments is the same specifically, as when first obtained. We have the following evidence that it is: Animals that had recovered from the infection with the virus after it had been cultivated in the guinea-pig for one year were immune to the inoculation of fresh virus obtained from man in the spring of 1907. Of two strains, one obtained in 1906 and one in 1907, each confers immunity against the other. This, of course, is independent of the possibility that the virus has undergone variations in the character of its pathogenicity as a result of prolonged cultivation in the guinea-pig.

The defibrinated blood of injected animals, the blood being drawn from the carotid artery or the heart, is the source of the virus, and when the term "virus" is

used, it is understood that it refers to such blood. The virus has been standardized as accurately as possible in terms of the minimum pathogenic dose. It is impossible to deal with the minimum lethal dose because of the fact that minimum infective doses are about as likely to prove fatal as several multiples of such doses. In most instances the minimum pathogenic dose lies between 0.01 c.c. and 0.03 c.c. The former frequently fails to produce infection whereas the latter is nearly always infective. For quantitative work it is important to draw the virus with respect to the duration of the infection, because of the possibility that antibodies which may appear later in the course may lessen infectivity. As illustrating the importance of this consideration, two strains were lost in the spring of 1906 by waiting until the animals had died before attempting to perpetuate the disease in succeeding generations.

We have gained the impression that virus which is taken rather late in the disease is more likely to produce infection, if it is diluted several fold with salt solution.

It has happened a number of times that small doses were infective whereas larger doses of the same virus were not. Such an experience occurred recently when infected blood from a horse, drawn rather late in the disease, was injected into two guinea-pigs in doses of 1 c.c. and 5 c.c.; the former amount caused spotted fever whereas the latter did not. This was not due to lack of susceptibility on the part of the latter animal, inasmuch as it contracted the disease later when inoculated with virus from the guinea-pig. We are inclined to the view that the failure of large doses to infect under these circumstances is due to the comparatively large quantity of antibodies in the larger quantities of blood. It is true that the proportionate quantity of antibodies to the number of microorganisms in 5 c.c. is the same as in 1 c.c. of blood, yet when the smaller quantity is used the antibodies are subject to greater dilution and it is in accordance with the known action of other antibodies to assume that their effect on the microorganisms would be somewhat attenuated in this instance by their dilution. Also there are good general grounds for believing that antibodies may be fixed or destroyed by the tissues to a certain extent, and if this is true the residuum of effective antibodies, in proportion to the number of microorganisms, would be lower when small quantities of virus are introduced than when larger quantities are given.

For most purposes the injections have been intraperitoneal, although the subcutaneous route has been employed for certain experiments.

#### CONCERNING IMMUNITY IN MAN.

In so far as we have been able to learn, there are no data regarding the immunity of man to Rocky Mountain spotted fever, although our knowledge of the matter is limited to the conditions in the Bitter Root Valley in Montana. There is no authoritative example of two attacks of the disease in the same person in this locality. It is true that one or more individuals state their belief that they have had more than one attack, but such statements lack the confirmation of the local physicians who are experienced in the recognition of the disease.

The results obtained in the monkey and the guinea-pig suggest that man probably acquires a strong immunity as a result of an attack.

This is a question, however, which can be approached from the experimental side by a study of the serum of those who have recovered.

#### ACTIVE IMMUNITY IN THE MONKEY.

The fact that one attack of spotted fever renders the monkey immune to a second inoculation, performed some months later, has already been reported by one of us.<sup>1</sup> An additional experiment illustrating the same point may be cited briefly.

On May 20, 1906, the monkey, a large *M. rhesus*, received intraperitoneally 10 c.c. of defibrinated blood from the patient, Porter. After an incubation period of about two days its temperature rose rapidly to 105.3 in the vicinity of which it remained for three days, and then gradually returned to normal during the course of five days more. The animal showed no eruption, although his eyes became much reddened. His blood was not inoculated into other animals in order to confirm the diagnosis of spotted fever and on this account there naturally existed a good deal of doubt as to whether the monkey had been infected with spotted fever by the inoculation.

On February 1, 1907, the monkey was given an immunity test, a control monkey (No. 24) being inoculated with the same quantity of virus. The inoculation caused no rise in the temperature of the animal, whereas the temperature of the control, after a brief incubation period, rapidly rose to 106.1, near which it remained until the animal was killed, on the sixth day after inoculation.<sup>2</sup> On the second day of high fever the control presented a roseolar eruption of the skin of the perineum, which was hemorrhagic at the time it was killed. The blood of this animal produced spotted fever in guinea-pigs.

The experiment reported previously and the one just cited indicate that an attack of spotted fever confers immunity in the monkey, which in the last instance was present in a marked degree for nine months. The quantity of virus injected was very large, consisting of defibrinated blood and a dense emulsion of the liver, spleen, and kidneys of an infected guinea-pig, a total quantity of 15 c.c.

<sup>1</sup> Ricketts, "Further Observations on Rocky Mountain Spotted Fever, etc.," *Jour. Amer. Med. Assoc.*, 1906, 47, pp. 1067-69.

<sup>2</sup> It was the custom at that time to kill the monkeys during the height of the fever in order to obtain quantities of virus for further experiments.

## ACTIVE IMMUNITY IN THE GUINEA-PIG.

In a previous article it was also stated that the guinea-pig that has recovered from spotted fever is thereafter immune to the disease, although the details of experiments were not given at that time.<sup>1</sup> This result has proved to be a rule to which no exception has been found among a great many tests. An illustrative experiment will be cited in this place and other instances are found through the remaining part of this report.

Guinea-pig 710 was inoculated with spotted fever by the larvae of an infected tick. Twelve days after the first larvae were seen to be attached, the temperature of the guinea-pig rose suddenly to 105.2, and on successive days was read at 105.3, 104.8, 104.3, 103.8, 102.3, and 102.2. This was a short course of fever and some doubt existed concerning the correctness of the diagnosis until the result of a transfer inoculation was known. On the day when the temperature of Guinea-pig 710 registered 104.3, 3 c.c. of blood were drawn from the heart and injected intraperitoneally into Guinea-pig 749. The latter passed through a typical course of spotted fever, the temperature being as follows on succeeding days after inoculation: 103.4, 103.4,—, 105.6, 104.8, 106.7, 106.4, 106, 104.6, 105.4, 104.4, 104, 104.3, 103, 103.5; recovery. The scrotum of 749 became hemorrhagic and sloughed in a typical manner, and the animal, furthermore, resisted a subsequent immunity test. After these experiments had proved beyond doubt that Guinea-pig 710 had been infected by the larval ticks, the animal was given an immunity test by the intraperitoneal injection of 2 c.c. of virus from Guinea-pig 781, which had been inoculated with the Eddy strain of the virus. On successive days the temperature of Guinea-pig 710 was as follows: 103.4, 103, 102.6, 102.4, 102.3, 102.5, 102.7, 103.1, 102.3, —, 103.6.

A good control for the experiment cited is found in the history of Guinea-pig 705 which also was used to determine whether or not the infected female tick transmits the disease to her progeny. Twenty-two days after the larvae of an infected tick were placed with the animal, the latter had four days of moderate fever: 104.3, 104.5, 103.1, 104.1, 102.1. This event suggested that the guinea-pig may have been given a mild infection by the larvae, but this was by no means certain

<sup>1</sup> Ricketts, *loc. cit.*

because the animals, confined and often exhausted as they are during such experiments, sometimes exhibit abnormal temperatures for one or several days which is entirely independent of infection with spotted fever. About three months later this animal received an injection of 2.5 c.c. of Bradley virus with the following result: 102, 102.5, 102.8, 105, 105.1, 104.7, 104.8, 103.9, 99.6; death. The figures represent the temperature of the animal on successive days. On the seventh day after inoculation the vulva became hemorrhagic; the autopsy showed typical changes in the spleen and lymph glands. Cultures were sterile.

It is a matter of first importance that a mild attack of spotted fever results in the formation of strong immunity. Usually, when small doses of virus are given, the animals either react with a typical and severe attack of spotted fever or they show no sign of infection whatever and in the latter instance it has been the custom to use them as duplicates in the course of routine passage. Occasionally, however, such animals have been found to be resistant to infection. For example, Guinea-pig 1064, which in a particular experiment had been injected with 0.01 c.c. of infected serum showed no disturbance other than one day of distinct fever, the following being the record of the temperature: 103.8, 102.8, 102.4, 102.8, 102.6, 104.8, 102.6, 103.2, 102.8, 103.2. Nine days after the first injection, it received intraperitoneally 1.25 c.c. of virus, diluted with salt solution, which had been kept in the ice-chest for five days. Although a control developed the disease, the animal was undisturbed by the inoculation, exhibiting normal temperature. A second immunity test was given 15 days later, with the same result.

One might be inclined to explain such occurrences by the assumption that occasional guinea-pigs are immune to the disease naturally. Two conditions, especially, argue against the correctness of this assumption: First we have found no guinea-pigs which are immune to the infection when inoculated with a moderate quantity of the virus taken from an animal early in the disease, the minimum quantity necessary for infection being in the neighborhood of 0.02 or 0.03 c.c. Second, it is not difficult to repeat the result just quoted by injecting suitable quantities of virus and immune serum, the existence of active immunity being proved by an immunity test which is given after the

passive immunity, established by the injection of the immune serum, has disappeared. This will be referred to again under the subject of "protective inoculation."

The active immunity is probably of long duration. Animals have been found to resist infection with excessive doses of virus more than a year after they suffered from the disease. The occurrence of hereditary immunity, to be referred to below, also indicates the profound change which the disease produces in the guinea-pig as well as the permanent character of the immunity.

There can be little doubt that the cause of the active immunity lies in the anti-infectious properties which may be demonstrated in the blood and serum of animals which recover, and perhaps also in the acquired power of the tissues of the immune animal to produce additional antibodies readily when fresh virus is introduced at a subsequent time. The properties of the serum are considered below under "Passive Immunity." At present we have no means of deciding whether the antibodies are antitoxic, germicidal or opsonic. Theoretically all three may be represented.

#### HEREDITARY IMMUNITY.

The offspring of a female guinea-pig that has recovered from spotted fever are endowed with a strong and protracted immunity. This has been demonstrated many times and the resistance is transmitted regardless of the degree of infection of the female parent, a fact which is shown in the case of certain "vaccinated" female parents which exhibited a minimum febrile reaction. The influence of the immune male in the transmission of the immunity has not been investigated. Illustrative experiments will be described.

The female guinea-pig 663 was infected by male tick No. 5 on June 30, 1907. The following was the course of the temperature: 101.6, 102.4, 102.2, 103.7, 104, 105.4, 105.4, 105.2, 104.9, 104.6, 104.7, 103.8, 102.7; recovery. She gave birth to one young on October 14. On November 27, when the young animal was about six weeks old, it was injected intraperitoneally with 1 c.c. of third-day virus (Bradley strain). It had been with the parent continuously until injected. The following daily temperatures were recorded: 103, 103.6, 103.9, 103.2, 103, 102.5, 103.4, 103.1, 103, 103.8, 103,



103.8, 102.6, 103.1, 102.7.<sup>1</sup> A control guinea-pig of the same age, from a normal female, inoculated with the same dose of virus, showed the following daily temperatures: 103, 102.8, 102.8, 105.6, 107, 106.3, 106, 104.4, 104.5, 103.2, 100.2; death. The anatomical changes at autopsy were typical of spotted fever.

This experiment is important as showing that a female may transmit the immunity to her young although her infection antedated the period of her pregnancy by several months; that is, the cells of the embryo were not stimulated to form protective substances by the presence of virus; only the germ cell could have been subjected to such an influence.<sup>2</sup>

The following "exchange experiment" indicates that the inherited immunity does not depend entirely on the milk which the young derive from the immune parent.

*Immune young which sucked immune parent.*—Guinea-pig 938, 30 days old. Sucked for 14 days, was then removed, and 16 days later was inoculated. Had no distinct rise in temperature. Guinea-pig 939, 34 days old. Remained with parent until inoculated. No distinct fever.

*Immune young which sucked normal parent.*—Guinea-pig 935, 29 days old. Remained with normal parent until inoculated. No distinct fever followed. Guinea-pig 936, 34 days old. Was with normal parent for 26 days. No fever followed inoculation.

*Normal young which sucked immune parent.*—Guinea-pig 937, 30 days old. Course of temperature following inoculation: 102.8, 102.4, 104.2, 105.3, 105.5, 105.6; death. Autopsy: typical of spotted fever.

*Normal young which sucked normal parent.*—Guinea-pig 940, 29 days old. Daily temperatures following inoculation: 102.4, 102.5, 103.8, 104.5, 105, 105.1, 98.7; death. Autopsy: typical of spotted fever.

The duration of the inherited immunity has not been definitely determined. In one instance it was present two and one-half months and in another three months after birth.

<sup>1</sup> In our experience the young guinea-pig frequently has a higher average temperature than the adult and it is subject to greater fluctuations.

<sup>2</sup> Spotted fever in the guinea-pig is strictly an acute infection. The blood of the animal which recovers is never infective for other animals, but on the contrary is protective, as stated later.

We have not yet studied the character of the inherited immunity. In the event that the infection of the parent occurred before her impregnation, there is reason to think that the immunity of the young is passive in character. If infection occurs during pregnancy, there may be opportunity for the establishment of active immunity on the part of the embryo. However, infection occurring at this time usually results in abortion. In the event that the condition is one of passive immunity, it differs from the passive immunity established by the injection of immune serum by its greater duration. The immunity conferred by the injection of 1 c.c. of immune blood into a guinea-pig has disappeared largely after the lapse of 30 days. It is to be noted, however, that the offspring of an immune female may contain in their body fluids a much larger quantity of protective substances than is introduced in 1 c.c. of immune blood, and that the elimination of the larger quantity may require a much longer time. This subject will be investigated further.

#### PASSIVE IMMUNITY.

The whole blood, defibrinated blood, and serum of animals that have recovered from spotted fever possess strong protective powers when injected with virus into healthy guinea-pigs. For the sake of convenience immune defibrinated blood, rather than serum, has been used for most of the experiments, although it has been determined that the protective powers lie in the serum. When the term "blood" is used, it refers to defibrinated blood.

For some of the experiments on passive immunity the blood of animals which have recovered and which have been subjected to no further treatment has been used; in other cases so-called "hyper-immune" blood was employed. This is the blood of guinea-pigs that after recovery received a series of injections of infected blood with the hope of increasing the quantity of protective substances. We doubt, however, whether such a practice is of much service in producing an increase in the amount of antibodies. In two instances the blood of guinea-pigs which were treated in this way showed an actual decrease in their protective power which fell below that of other immune animals that had not received fresh injections of virus. Both of these animals were bled repeatedly during the immunizing

process, however, and the decrease may have been due to the dilution accompanying the restitution of the blood. As a rule, the blood of guinea-pigs that have recovered recently has shown as great protective powers as that of "hyper-immune" animals. It is not unlikely that the concentration of antibodies in the blood of the immune animal decreases with time. There is a possible source of error in determining this point, however, in that a number of successive bleedings of the same guinea-pig brings about a dilution of the protective substances in his body simulating a decrease by elimination or destruction within the body. Experiments bearing on this point have not been brought to completion.

The blood of guinea-pigs that have recovered from the disease recently protects in doses of from 0.1 to 0.3 or 0.4 c.c. against infection with 1 c.c. of third-day virus, a quantity which represents at least from 30 to 60, and in some instances 100, minimum pathogenic doses. This result is obtained when the immune blood and virus are mixed before injection, the inoculations being intraperitoneal. Preliminary experiments indicate that the protection is not so pronounced when the two are injected into different parts of the body at the same time, although the difference is not great.

The following experiment gives an approximate idea of the duration of the passive immunity when 1 c.c. of immune blood is injected subcutaneously (Table 1). The immune blood in this instance was a

TABLE 1.  
DURATION OF PASSIVE IMMUNITY.

Guinea-pig	Interval between Injection of Serum and Virus	Quantity of Virus Injected	Result
1001.....	20 days	1.0 c.c.	No fever
1000.....	33	1.0	Slight fever for 5 days
1002.....	35	1.0	Moderate fever for 5 days
1003.....	45	1.0	Severe fever for 6 days
1004.....	49	0.5	Severe course of fever. Killed
1005.....	55	0.03	Became infected after an incubation period of one week

mixture, obtained from three immune guinea-pigs (862, 878, 778), two of which had recovered from spotted fever one month previously and the third about three months previously. One-tenth cubic centimeter protected against 1 c.c. of third-day virus in controls, the mixtures being injected intraperitoneally. The experiment animals

received the virus subcutaneously at different periods following the injection of the immune blood.

The relation of the duration of the passive immunity to the quantity of immune serum injected has not been determined.

The converse of this experiment was performed also; a number of guinea-pigs were inoculated subcutaneously with 1 c.c. of virus each, and the subsequent period determined at which 1 c.c. of immune blood would prevent the development of spotted fever (Table 2). In this case the immune bloods were drawn immediately before injection and they came from different immune animals; hence they probably were not of uniform value, though certainly not differing greatly.

TABLE 2.  
PROPHYLACTIC EFFECT OF IMMUNE BLOOD WHEN INJECTED SUBSEQUENT TO VIRUS.

Guinea-Pig	Interval between Injection of Virus and Immune Blood	Result
851.....	1 day	One day of distinct fever which may have been due to other causes
852.....	2 days	The same result, the single day of fever occurring on the same day
853.....	3	The same result
854.....	4	Three days of high fever, reaching 106.5, followed by three days of low fever (about 104)

The experiment shows protection against 30 to 60 pathogenic doses at the least, when 1 c.c. of immune blood is given three days after the injection of the virus. From this time the injections of immune blood could be considered to have only a curative effect, and this will be mentioned later.

The results of these experiments on protection by means of immune blood or serum, of which we have abundant confirmation, would seem to have an important practical bearing. There is good reason to believe, on the basis of the results obtained, that serum prophylaxis of man is feasible provided sufficient quantities of a serum of reasonable strength can be prepared. Manifestly it would operate successfully only in case the inhabitants of infected districts who are bitten by ticks would report for prophylactic injections within two or three days after receiving the bite. If the results obtained with the guinea-pig apply also to man, the serum when given in sufficient quantity would ward off the danger pertaining to the recent exposure, but probably could

not be considered protective for a period longer than three or four weeks. A subsequent exposure would require another injection of serum.

We have been able to infect the horse with spotted fever recently and are now engaged in a study of the protective power of the serum obtained after the recovery of the animal. Preliminary experiments indicate that its value differs little from that of the guinea-pig, hence there is reason to believe that a protective serum in desirable quantities will be available.

#### AN EXPERIMENT IN SERUM THERAPY.

We have made preliminary observations on the curative value of the immune blood from the guinea-pig and shall describe an experiment which seems to indicate that its therapeutic power is not of high degree (Table 3).

This is a continuation of the passive immunity experiment described on p. 232, in which equal quantities (1 c.c.) of virus were inoculated into a number of guinea-pigs and the effect of the immune blood noted when given at subsequent periods. Inasmuch as the remaining animals began to show fever on the fourth day after inoculation, it became thereafter an experiment to determine the curative value of the immune blood.

It had been determined in other experiments that the normal blood of the guinea-pig has no protective power against spotted fever. Thus Guinea-pig 884 received intraperitoneally a mixture of 1 c.c. of normal blood and 0.3 c.c. of third-day virus and died of spotted fever after nine days. Also Guinea-pig 886 received 1 c.c. of normal blood and 0.05 c.c. of virus and exhibited the course characteristic of spotted fever, including hemorrhage into and sloughing of the scrotum. It resisted an immunity test given two months after recovery.

Inasmuch as both controls in this experiment recovered, the influence of the immune blood can be determined only by its effect on the duration and severity of the fever, the normal duration of which in this instance was approximately six days. With this as a basis of comparison, it is seen that 2 c.c. of immune blood given on the first day of fever exercised but slight if any influence on the course. Five cubic centimeters given on the first day quite positively shortened the course

since there were only three days of severe fever. One cubic centimeter given daily had no pronounced influence, although the temperature for the last three days was lower than in the controls. Two cubic centimeters given daily beginning with the second day of fever may have been a factor in the lower temperature during the last three days.

TABLE 3.  
THE CURATIVE POWER OF IMMUNE BLOOD.

Virus injected December 4, 1907, subcutaneously.

Injections of immune blood subcutaneous on dates indicated.

Guinea-pigs designated by numbers, 855, etc.

Dec.	Temp. 855	Immune blood	Temp. 856	Immune blood	Temp. 857	Immune blood	Temp. 858	Immune blood
5.....	103.8		103.4		104.		103.9	
6.....	103.4		104.		103.7		103.4	
7.....	103.5		104.2		104.9		104.8	
8.....	103.6		103.		103.7		103.1	
9.....	105.6	2 c.c.	105.8	5 c.c.	105.	1 c.c.	105.8	
10.....	105.5		105.		105.7	1	106.	2 c.c.
11.....	105.7		105.		106.3	1	105.7	2
12.....	104.5		103.4		104.8	1	104.6	2
13.....	104.2		103.9		104.2	1	104.2	2
14.....	.....		.....		104.	1	104.8	2
15.....	102.2		102.5		102.9		103.9	
16.....	102.3		102.3		103.1		103.6	
17.....	102.2		102.		102.5		103.5	
18.....	102.6		102.6		103.7		103.3	
19.....							103.	
20.....	Recovery		Recovery		Recovery		102.3	
							Recovery	

Dec.	Temp. 859	Immune blood	Temp. 860	Immune blood	Controls*	
5.....	103.3		103.4		103.9	103.6
6.....	104.		103.7		102.7	104.
7.....	104.4		104.2		104.	104.
8.....	103.7		103.6		103.5	103.7
9.....	105.6		104.3		105.2	106.4
10.....	106.4		106.4		106.3	106.2
11.....	106.8	2 c.c.	106.2	3 c.c.	106.	105.7
12.....	106.	2	105.2	3	105.4	105.1
13.....	105.8	2	105.3	3	105.6	105.8
14.....	105.2	2	105.8	3	.....	.....
15.....	103.6		103.9		103.2	103.
16.....	103.2		104.		103.4	103.5
17.....	102.7		103.1		103.	103.
18.....	102.8		102.7		102.7	103.1
19.....			103.3			
20.....			103.			
	Recovery		Recovery		Recovery	Recovery

\* Inoculated with 1 c.c. of virus alone.

The same amount given daily, beginning on the third day of fever, had no effect, and a similar result is noted when the amount was increased to 3 c.c. daily.

The experiment cited seems to show quite positively that the immune blood exercises little influence on the disease unless it can be

given early and in large quantities. An important practical difficulty arises here, for the disease in man is rarely recognized until the eruption appears, which may be at any time from the second to the fifth day of fever. Physicians who are experienced in the diagnosis of the infection often make a probable diagnosis of spotted fever on the first or second day of sickness, in the spring of the year, in the case of patients that reside in infected districts. The probable diagnosis should be facilitated if a history of tick bite within one to two weeks preceding the onset of symptoms is given. In such cases, a large quantity of the serum given immediately may exercise a modifying influence on the course of the infection; in the event that the probable diagnosis was incorrect, there would be no likelihood of harm as a consequence of the injection of the serum.

The curative value of the immune serum from the horse is being studied carefully; inasmuch, however, as the preliminary tests have shown that it possesses about the same power as the blood of immune guinea-pigs, it is not anticipated that the results will be very different.

It is not probable that a serum of greater power than that afforded by the animal which has recovered from spotted fever, will be obtained until the microorganism can be grown in desirable quantities in artificial cultures. During the course of the infection there is already in the animal's body such a quantity of the organism that 0.02 or 0.03 c.c. of his blood is infective, and at present there is no means of increasing this amount for subsequent injections.

#### PROTECTIVE INOCULATIONS.

For more than a year occasional attempts have been made to attenuate the virus of spotted fever by desiccation, by the addition of glycerin, and by means of heat, so that it would be suitable for vaccination. Desiccation for this purpose has proved to be utterly unreliable and has been abandoned. The experiments will not be described further than to say that the principle followed was to begin the injections with comparatively large quantities of virus that had been dried in a vacuum over sulphuric acid for a longer period than was required to kill all the organisms, and for succeeding injections to use smaller quantities of virus which had been dried for shorter periods, passing finally to minute amounts of fresh virus. Infection eventually

resulted in practically all the animals, and although it may in the end be possible to produce immunity in this way, the method is so tedious, long, and uncertain, that it appears to have only theoretical interest.

The value of glycerin and low degrees of heat as attenuating agents is still being studied.

No systematic attempt has been made to determine the vaccinating properties of minute doses of the virus. As a rule a minute quantity either produces frank infection or causes no disturbance whatever, and in the latter case the animals are, in nearly all instances, susceptible to reinoculation. Occasional exceptions have been encountered, however. Such an animal (Guinea-pig 1064) was referred to under the discussion of active immunity (p. 227). In this instance marked active immunity developed, although the animal had but one day of fever. On account of the uncertainty as to what the virus will do when injected in quantities which approximate the minimum pathogenic dose, it is manifest that minute doses cannot be utilized for practical vaccination.

On account of the protective power which immune blood possesses, the possibility of mixed immunization or "sero-vaccination" seemed promising and we have devoted some time to the study of this subject.

At the beginning of the work three plans of inoculation were adopted: First, the immunizing effect of a single mixture of immune blood and virus. Second, the effect of repeated injections given at short intervals, the ratio of the quantity of the immune blood to the virus being gradually decreased until pure virus was injected. Third, the same as the second plan, except that longer intervals were allowed to intervene between the injections. The ultimate object in all three methods was to establish active immunity without causing severe infection. This was found to be readily possible with regard to the second and third methods, although the development of immunity in most instances was characterized by a mild and brief febrile reaction some time during the course of the injections. These methods, however, are protracted and tedious and are more of theoretical than practical interest, particularly since a high degree of immunity can also be established by a single injection of virus and immune blood when the two are used in proper proportions.

We shall therefore limit our description to certain experiments in



which immunity was established in one instance by two (Table 4), and in another by one, injection.

TABLE 4.  
THE IMMUNIZING EFFECT OF TWO INJECTIONS.

First injection, consisting of virus mixed with different quantities of immune blood. Injections intraperitoneal. Immune blood from two animals (765, 766) that had recovered spontaneously.

No. of Experiment Animal	Virus	Immune Blood	Result
789.....	1 c.c.	1.5 c.c.	No fever
790.....	1	1.0	" "
791.....	1	0.7	" "
792.....	1	0.3	" "

Second injection, consisting of 1 c.c. of virus, given 25 days after the first.

Date	Guinea-pig 789	Guinea-pig 790	Guinea-pig 791	Guinea-pig 792
Dec. 2.....	102.0	102.2	102.2	102.4
3.....	102.3	103.	103.	103.
4.....	102.4	102.6	102.7	102.8
5.....	102.1	102.6	102.3	103.
6.....	101.8	102.6	102.4	104.7
7.....	103.6	103.4	103.	105.5
8.....	102.8	103.8	103.4	104.
9.....	102.8	103.2	104.2	104.8
10.....	103.	103.4	103.9	103.3
11.....	103.8	103.7	104.	94.4
12.....	102.7	102.7	102.2	Death

Following the second injection all the animals showed a certain amount of febrile reaction, although in the first three the rise in temperature was no greater than often encountered from other causes, such as minor injuries to the colon by the thermometer.

It was attempted to learn the degree of immunity which had been established in 789, which showed a minimum reaction, and in 791 which showed a more pronounced though mild reaction, by testing the protective power of their defibrinated bloods, in two consecutive experiments (Tables 5 and 6).

The first test, shown in Table 5, is not very satisfactory, particularly regarding the animals injected with the blood of Guinea-pig 791. Guinea-pig 960 which received the largest dose of this blood (1 c.c.) ran a distinct course of fever, the cause of which may not be open to determination. When sufficient time has elapsed for the passive immunity, which was conferred by the immune blood, to be eliminated, the protective power of its blood will be ascertained, and if this proves to be of sufficient strength it will have to be concluded that the animal suffered from a moderately severe attack of spotted fever, in spite of

TABLE 5.

THE PROTECTIVE POWER OF THE BLOODS OF THE VACCINATED GUINEA-PIGS 789 AND 791.

Test 1, performed 25 days after the second injection.

Virus, 1 c.c. + varying quantities of immune blood.

IMMUNE BLOOD FROM GUINEA-PIG 789				IMMUNE BLOOD FROM GUINEA-PIG 791			CONTROLS, RECEIVING 1 C.C. OF VIRUS EACH†	
Date	1 c.c. No. 957	0.7 c.c. No. 958	0.3 c.c. No. 959	1 c.c. No. 960	0.7 c.c. No. 961	0.3 c.c. No. 962	No. 965	No. 966
Dec. 27.....	102.8	102.8	103.0	103.0	102.8	102.7	102.4	103.6
28.....	102.9	103.1	103.2	103.5	103.3	102.8	102.6	103.8
29.....	102.6	103.	104.3	103.8	104.	102.8	102.4	102.8
30.....	102.6	103.6	105.2	104.	104.	103.	103.	103.2
31.....	102.4	103.5	103.9	103.5	103.4	103.1	104.4	104.
Jan. 1.....	.....	.....	.....	.....	.....	.....	105.2	105.4
2.....	102.4	103.	104.8	104.7	103.2	102.7	Killed	Killed
3.....	.....	.....	.....	.....	.....	.....	.....	.....
4.....	103.2	104.4	104.	104.3	103.2	102.8	.....	.....
5.....	102.8	104.3	102.8	104.5	102.5	102.4	.....	.....
6.....	.....	.....	.....	.....	.....	.....	.....	.....
7.....	102.6	104.	103.6	104.	102.3	102.2	.....	.....
8.....	102.4	103.4	104.3	104.7	102.4	102.4	.....	.....
9.....	101.6	103.5	103.2	104.8	102.3	102.6	.....	.....
10.....	103.2	103.1	104.3	103.9	103.	103.4	.....	.....
11.....	.....	103.5	104.	102.8	.....	.....	.....	.....
	Recovery	Recovery	Recovery*	Recovery	Recovery	Recovery		

\* Guinea-pig 959 died two weeks later, showing pneumonia and cheesy abscesses.

† Guinea-pigs 965 and 966 were "passage" animals and they were killed on January 2 in order to perpetuate the strain in other guinea-pigs.

TABLE 6.

THE PROTECTIVE POWER OF THE BLOODS OF THE VACCINATED GUINEA-PIGS 789 AND 791.

Test 2, performed 20 days later than the first test, and 54 days after the second immunizing injection was given.

Virus, 1 c.c. + varying amounts of the immune bloods.

DATE	IMMUNE BLOOD FROM GUINEA-PIG 789			IMMUNE BLOOD FROM GUINEA-PIG 791			CONTROLS = VIRUS ALONE	
	0.6 c.c. No. 1074	0.4 c.c. No. 1073	0.2 c.c. No. 1072	0.5 c.c. No. 1077	0.3 c.c. No. 1076	0.1 c.c. No. 1075	0.01 c.c. No. 1078*	0.05 c.c. No. 1079
Jan. 25.....	103.	103.8	103.6	102.8	102.6	103.2	102.8	103.2
26.....	102.8	103.2	103.	103.	101.8	102.	102.4	102.4
27.....	103.	103.2	102.6	102.6	102.2	102.	102.2	102.2
28.....	102.8	103.2	102.6	102.6	102.8	103.4	102.4	104.
29.....	102.6	102.4	102.	102.8	102.6	102.4	100.8	104.6
30.....	103.4	103.2	105.	102.8	103.2	.....	102.	105.2
31.....	103.4	104.4	104.8	.....	103.2	.....	102.4	105.4
Feb. 1.....	104.	104.2	105.6	103.6	102.8	105.	103.2	105.8
2.....	104.4	105.3	104.4	102.7	103.8	104.3	102.4	105.2
3.....	103.6	104.8	104.	102.8	104.	103.8	102.4	104.
4.....	105.2	105.	104.4	102.	104.6	104.	103.2	99.8
5.....	104.4	104.	104.6	103.	104.	104.	103.4	Death†
6.....	104.4	103.4	104.4	102.8	103.4	102.8	103.6	.....
7.....	102.4	103.6	103.8	102.8	102.8	102.	102.2	.....
8.....	102.2	102.8	102.8	102.2	103.	102.	102.4	.....
9.....	102.4	102.	102.8	102.3	102.6	102.3	102.4	.....
	Recovery	Recovery	Recovery	Recovery	Recovery	Recovery	Recovery	.....

\* Guinea-pig 1078 became infected when an immunity test was given on February 28, hence in this experiment the minimum pathogenic dose was greater than 0.01 c.c. of virus, but equal to or less than 0.05 c.c. as shown by the fatal course in Guinea-pig 1079.

† The genitalia were hemorrhagic, and the spleen was greatly enlarged.

the large dose of protective blood which it received, and in spite of the fact that one-third of this dose protected Guinea-pig 962.

Having in mind the animals which received 0.7 and 0.3 c.c. of the two immune bloods, the impression is given that the blood of 791 was somewhat more protective than that of 789.

The difference in the protective power of the two bloods is manifest in the second test. Thus, 0.5 c.c. of the blood of 791 prevented fever entirely, whereas 0.6 c.c. of 789 did not prevent the development of a distinct and rather high course of fever; 0.3 c.c. of the former permitted moderate fever for five days, while 0.4 c.c. of the latter allowed a marked febrile course of nine days; and the conditions are similar in regard to the third animal of each group (1075 and 1072). One probably could not come nearer to a comparison than to say that 0.6 c.c. of the blood of Guinea-pig 789 was equal to 0.1 c.c. of that from 791, since the severity and duration of the fever of the corresponding animals were about the same.

Comparison of the first test with the second indicates that the protective substances underwent a diminution in quantity in the interval of 29 days. In the first test the blood of Guinea-pig 791 in a dose of 0.3 c.c. prevented fever entirely, with the possible exception of two days when the temperature was 103 and 103.1 respectively. In the second test the same quantity of blood permitted a distinct though moderate fever for five days. Similarly, 0.3 c.c. of the blood of Guinea-pig 789 in the first test afforded greater protection than 0.6 c. c. in the second test. The conclusion that such a diminution does occur has been borne out in other experiments.

Such comparative experiments, performed at different times, are subject to possible error in that the minimum pathogenic dose of two different lots of virus may not be identical. In our experience, however, this variation has been slight and certainly would not cause greater error than would result from slight differences in the susceptibility of different animals.

An experiment similar to the one just described was carried on with Guinea-pigs 835, 836 and 837, only the essential points of which will be given.

On November 25, all three were injected with 1 c.c. of virus to

which had been added different quantities of immune blood; for 835, 0.3 c.c.; 836, 0.1 c.c., and 837, 0.05 c.c.

Guinea-pig 835 had no fever following the injection. Guinea-pig 836 showed the following course of temperature on successive days: 103.4, 103.7, 103, 103, 102.9, 103.8, 103.4, 103.8, 104.4, 104.5, 103.3, 102.8, 103.2, 102.6. The fever traceable to the virus was brief and mild in character. Guinea-pig 837 suffered severe infection with the following course of the temperature: 102.9, 102.8, 102.9, 103.1, 102.8, 105, 105.6, 105.8, 104.9, 104.6, 105.1, 104.2, 103.4, 103, 103.3, 102.6, 102.6.

They received no further inoculations of virus, hence the experiment represents an attempt to immunize by one injection.

The first comparative test of the protective powers of the bloods of the three animals was made on January 7, about two months after the injections were made. This was ample time for all the passive immunity to have disappeared, as shown by an experiment quoted earlier in this paper. Suitable quantities of the different bloods were not used to afford intelligent comparison of their properties. The second test, performed February 5, showed that 2 c.c. of the blood of 835 had no protective effect, whereas 0.8 c.c. from 836, and 0.5 c.c. from 837 showed moderate protection. In the meantime an immunity test was given to 835, which resulted in its infection with spotted fever, hence it may be concluded that the initial injection, the immunizing (?) injection, which was followed by no fever, had produced little or no immunity in this animal.

A third test of the blood of 836 and 837 showed that 0.9 c.c. from 836 did not afford complete protection, whereas 0.5 c.c. from 837 entirely prevented the development of fever with the dosage of the virus used, namely, 1 c.c. of third-day virus. This test was made three months after the immunizing injection was given.

Both of the experiments described indicate that there is a parallel between the severity of the reaction following the immunizing injection and the subsequent protective power of the blood, and it is reasonable to suppose that the degree of immunity conferred by the vaccination corresponds to a certain extent with the concentration of the protective substances which appear in the blood. This does not mean, however, that a severe or even moderate febrile reaction is necessary in

order that distinct immunity be conferred. It has happened frequently that a barely perceptible reaction is followed by the development of pronounced resistance. Two instances may be cited in which the immune blood was given a few days in advance of the virus.

On December 1, 1907, Guinea-pigs 844 and 845 received each 1 c.c. of immune blood subcutaneously. Three or four days later, respectively, each was given 1 c.c. of third-day virus (Eddy strain) subcutaneously. The temperature of Guinea-pig 844 was as follows: 102, 102.8, 103.4, 103.3, 103.2, 101.8, 103.3, 103.2, 102, 102.7. That of Guinea-pig 845 was: 102.9, 103.4, 102.7, 103.3, 101.7, 102.7, 102.9, 102.5, 102.9. Sixty-seven days later each received intraperitoneally 1 c.c. of third-day virus, one being given the Eddy and the other the Bradley strain. Neither showed the slightest febrile disturbance. Controls developed the disease typically.

#### HEREDITARY IMMUNITY IN VACCINATED ANIMALS.

Inasmuch as strong immunity may be produced in guinea-pigs by the method of "sero-vaccination," in which only a minimum febrile reaction is necessary, it would be expected that the immunity conferred on females in this way would be conveyed to their offspring. This would be anticipated from the fact that such a transfer occurs in the case of females which have suffered from a severe attack of the disease, as described on a previous page.

This phenomenon was noted in relation to Guinea-pig 791, whose history has been given already, and to Guinea-pig 794, which had been immunized by a series of graded injections in which the quantity of immune serum was reduced until pure virus was administered.

Two young were born to Guinea-pig 791 on December 30, 1907. This was approximately one month after she received the second injection, consisting of virus alone and about two months after the mixed injection was given. One of the young (No. 1080) when 25 days old received 0.5 c.c. of virus intraperitoneally. Its temperature and appearance were unchanged by the injection, whereas a control of the same age (No. 1087), which received the same dose died of spotted fever 10 days later. The remaining animal (1081), when 52 days old was given an intraperitoneal injection of 1 c.c. of virus. No fever resulted and the guinea-pig is still living; the control acquired a

severe infection. The same result was obtained with one of the young of Guinea-pig 794, 24 days after its birth.

The experiments quoted and others which it seems unnecessary to describe show that successful sero-vaccination of the guinea-pig is possible. We are studying this problem further to determine the applicability of the method to man, paying particular attention to methods of standardization of the virus and immune serum, the durability of the protective substances under different conditions of preservation and the constancy with which a desired reaction can be obtained. It is our intention at the same time to investigate the behavior of the monkey to this method of vaccination.

It does not follow that one can pass directly from the guinea-pig to man or from the monkey to man in sero-vaccination, using corresponding proportions of virus and immune serum. The unknown susceptibility of man in comparison with that of the monkey and guinea-pig is a serious stumbling-block in this connection. A mixture which is neutral for the guinea-pig or which produces only a slight reaction in it, may produce a severe reaction in man. The converse may also be true: that a dosage or a proportion of constituents which would excite an immunizing reaction in the guinea-pig or monkey would be without effect in man. There is also a further possibility to face, namely, that the virus, as a result of cultivation in the guinea-pig, may have undergone modifications in its virulence, whereby it may have become less virulent or more virulent for man.

Only one method could possibly be advocated at the outset; namely, to use such proportions of virus and immune serum as would leave no question as to the safety of the procedure, assuming for the time that the virus has the greatest possible virulence for man. An index of the effect of such injections could be obtained by studying the properties of the resulting serum. In the event that no antibodies were formed it would then appear justifiable to decrease the proportion of immune serum to that of virus, again studying the effect of the injection on the properties of the serum. This process could be continued until a mixture is obtained which causes the appearance of antibodies without exciting a severe reaction.

## SUMMARY.

An attack of spotted fever in the guinea-pig and monkey produces a strong active immunity of long duration. This immunity is characterized by the presence of protective antibodies in the serum which may be demonstrated by injecting mixtures of virus and immune serum. The concentration of the antibodies in the blood of the immune animal undergoes a decrease in the course of several weeks.

The female that has recovered from spotted fever transmits immunity to her young. The young are immune even when the female acquired her immunity several months before impregnation. The immunity of the young does not depend on the ingestion of milk from the immune mother. The character of the inherited immunity has not yet been determined, although it is presumptively a passive immunity that differs from the passive immunity conferred by the injection of immune serum by its longer duration. The long duration of the inherited immunity may depend on the longer time required for the elimination of large quantities of protective substances.

Passive immunity may be established in the healthy guinea-pig by the injection of blood or serum from the immune guinea-pig. The immune defibrinated blood contains antibodies in such concentration that 0.1 c.c. often protects against 1 c.c. of third-day virus, representing anywhere from 30 to 100 minimum pathogenic doses. In other instances 0.3 or 0.4 c.c. of immune blood are required for this degree of protection. When 1 c.c. of strong immune blood is injected subcutaneously into healthy guinea-pigs, the passive immunity is still present in marked degree after 20 days; after 38 days it is present only in such degree that a mild course of spotted fever results when virus is injected; after 45 days it is no longer manifest. It is possible that passive immunity would not last so long if the immune blood is injected into a foreign species.

The guinea-pig may be protected against spotted fever following its inoculation with infected blood, provided the immune blood is administered on the second or third day after inoculation.

The curative power of the immune blood or serum is low and in order to produce a distinct effect it is necessary to begin its administration early in the disease and to give relatively large quantities. It exerts

a modifying effect on the severity of the infection without bringing about rapid subsidence of the symptoms.

By the method of mixed immunization or sero-vaccination, in which virus and immune blood or serum are mixed in suitable proportions, it is possible to immunize the guinea-pig by one or several injections, with the result that he is thereafter immune to infection. The blood of animals immunized in this way contains protective antibodies in fairly high concentration and in the case of females the immunity is transferred to the offspring. The quantity of antibodies produced by this method of immunization probably is in proportion to the severity of the febrile reaction which follows the administration of the immunizing dose. However, strong immunity has resulted in some instances in which the immunizing injection caused only a barely perceptible febrile reaction.

It may be possible to use the immune serum from the horse, which we have shown to be susceptible to inoculation, for the prevention of the disease in man. For this purpose the serum should be injected in sufficient quantity within two or three days following the bite of the tick. Such an injection should not be considered protective for a longer period than three weeks.

The method of sero-vaccination is not yet sufficiently perfected to warrant its application to man, but the subject is being studied further in order to determine its safety and efficiency.

There is no hope of obtaining a stronger serum for curative purposes than that yielded by an animal which has recently recovered from the disease, until the microorganism can be cultivated artificially, thus making available a desirable quantity of antigens for immunization. Even with this condition realized the results of further immunization cannot be anticipated but must await experimental determination.



# ON THE ACTIVATION OF OPSONIN IN HEATED HUMAN SERUM.\*

## MODIFICATION OF WRIGHT'S HEATED SERUM TEST FOR TUBERCULOSIS.

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WITH the idea of showing whether any relation could be demonstrated between the thermostabile (found in infected cases) component of opsonin and that of complement, some experiments were undertaken which have at this stage resolved themselves into what might be termed a modification of Wright's Heated Serum Test.

Wright's Heated Serum Test is briefly as follows: Both the immune and normal sera are heated for 10 minutes at 60° C. and the phagocytic index taken. It will usually be found that the sera from cases infected with tuberculosis retain their phagocytic power in marked contrast to the heated normal sera whose indices are usually nothing or at most found in the second decimal place (as 0.05). Thus there is induced in the serum of the patient undergoing auto-inoculation a thermostabile substance capable of producing phagocytosis. Occasionally this substance is not demonstrable in undoubted cases. This loss of phagocytosis was investigated by adding a fourth volume of complement (in the form of fresh diluted rabbit serum). Under these conditions the sera from infected cases gave a diagnostic phagocytosis while the loss of phagocytosis of normal sera was unaffected. This is shown in the following experiments in which the serum used was heated to 60° C. for 10 minutes, the complement being rabbit serum diluted 1:12.

### EXPERIMENT 1.

	Phagocytic Index of Heated Serum Alone	Phagocytic Index of Heated Serum + Complement	Phagocytic Index of Heated Serum + Salt (1.5%)
Patient A.....	0.05	0.28	....
" B.....	0.02	0.22	0.03
" C.....	0.42	....	....
Normal a.....	0.00	0.01	0.04
" b.....	0.04	0.05	....
R. S. 1 <sub>2</sub> .....	0.04	....	0.04

\* Received for publication March 2, 1908.

## EXPERIMENT 2.

	Phagocytic Index of Heated Serum Alone	Phagocytic Index of Heated Serum + Complement
Patient D.....	0.02	0.18
" E.....	0.21	0.21
" F.....	0.04	0.03
Normal a.....	0.03	0.03
" b.....	0.03	0.05
R. S. $\frac{1}{12}$ .....	0.01	....

## EXPERIMENT 3.

(In this the sera were heated at 56° for 30 minutes.)

	Phagocytic Index of Heated Serum + Complement
Patient G.....	0.28
" H.....	0.09
" I.....	0.17
" J.....	0.40
" K.....	0.18
" L.....	0.01
Normal a.....	0.05
" b.....	0.05
R. S. $\frac{1}{10}$ + salt (1.5%).....	0.07

## EXPERIMENT 4.

The sera were heated for 20' at 60° on the same set as in Experiment 5. This exposure destroyed the phagocytosis in all sera, no count being more than 0.04 as given by the diluted rabbit serum.

## EXPERIMENT 5.

	Phagocytic Index of Serum Alone	Phagocytic Index of Serum + Com- plement
Patient M.....	....	0.14
Normal a.....	....	0.02
" c.....	....	0.02
R. S. $\frac{1}{10}$ .....	0.02	....

In all the experiments especial care was taken in the preparation of the tubercle emulsion (dried bacilli being ground up with 1.5 per cent salt solution and allowed to sediment in long tubes. The resulting emulsion happened to be free from clumps and gave a normal count of about 1.30 in a 20-minute incubation period. The same emulsion was used in all tests.

Several of the results in the experiments given have been confirmed with the same sera two or more times. In one instance a contradictory count was obtained. This has not been accounted for.

The clinical diagnosis in the cases utilized in testing for tuberculosis is as follows:

*Patient A.*—Pulmonary tuberculosis which at the time of the test showed a slight rise in temperature.

*Patient B.*—Advanced case of pulmonary tuberculosis.

*Patient C.*—Pulmonary tuberculosis which was at the time doing well and showed normal temperature.

*Patient D.*—Periorbital abscess, suspicious Calmette reaction.

*Patient E.*—Early pulmonary tuberculosis, positive Calmette reaction.

*Patient F.*—Lobar pneumonia.

*Patient G.*—Interstitial keratitis, positive Calmette reaction.

*Patient H.*—Rhinitis and laryngitis, negative Calmette test.

*Patient I.*—Tuberculous hip disease, positive Calmette reaction.

*Patient J.*—Tuberculous hip and double psoas abscess, positive Calmette reaction.

*Patient K.*—Cervical adenitis, with sloughing, negative Calmette test.

*Patient L.*—Anemia, negative Calmette test.

*Patient M.*—Tuberculosis of knee of two years' duration without much change.

Among these cases, Patient D shows a positive count of 0.18 and a suspicious Calmette reaction; Patient K, a negative Calmette and a positive count. Patient L, diagnosed as tuberculosis is the lowest count, being 0.14 with the R. S. 0.01; 0.05 is the highest count obtained from a normal.

In view of the fact that the detection of the specific amboceptor (as utilized by Wassermann, Plaut and others) is of slighter diagnostic value in tuberculosis than in syphilis, pertussis, gonorrhea, etc., some experiments were undertaken on four cases of gonorrheal arthritis, available at the time.

#### EXPERIMENT 6.

	Phagocytic Index of Heated Serum + Complement	Phagocytic Index of Heated Serum + Salt Solution
Patient A.....	2.06	1.90
" B.....	3.36	....
Normal a.....	0.80	0.76
" b.....	0.27	....

#### EXPERIMENT 7.

	Phagocytic Index of Heated Serum + Complement
Patient A.....	5.78
" C.....	5.00
Normal a.....	2.46
" b.....	2.82
R. S. $\frac{1}{4}$ .....	1.94

#### EXPERIMENT 8.

	Phagocytic Index of Heated Serum + Complement
Patient D.....	1.28
Normal a.....	0.73
" b.....	0.68
R. S. $\frac{1}{10}$ .....	0.74
Unheated Serum of Nor. A....	2.00

In the estimation of the index to the gonococcus emulsion a five to six hours' growth incubated for seven to eight minutes was employed.

Of the four cases of gonorrheal arthritis utilized two (A and B) were at the time being treated with killed gonococci, the remaining two (C and D) were under ordinary treatment without inoculation. Patients A, B, and C were diagnosed clinically as gonorrheal arthritis before the test; the fourth case (Patient D) has subsequent to the laboratory diagnosis been accepted as gonorrheal in origin.

In the results given no attempt has been made at completeness. They have to a considerable extent been obtained in a series which (not yet complete) was undertaken to show the specificity of opsonin to be closely bound up with the thermostabile part, and to adduce experimental evidence supporting a treatment of bacterial diseases with both Wright's vaccines and serum.

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## ON THE SPECIFICITY OF OPSONINS IN NORMAL SERUM.\*

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NORMAL serum contains opsonin for many different kinds of bacteria and of red corpuscles. The question whether this wide range of opsonic action is dependent on a common opsonin or on several more or less specific opsonins has been answered differently by different investigators. Thus Bulloch and Western<sup>1</sup> who were the first to study this question, drew the following conclusion from their absorption experiments: "When staphylococci are brought into contact with normal human serum and are subsequently removed by centrifugalization, the serum loses its opsonic power for staphylococcus, although the opsonic power for *Bacterium pyocyaneum* is preserved.

Contact of normal human serum with tubercle bacilli leaves the opsonic power of that serum for staphylococcus almost intact, while the opsonic power for tubercle bacillus is completely removed.

Contact of normal human serum with staphylococcus leaves the opsonic power of that serum for tubercle bacillus almost intact, while the opsonic power for staphylococcus is completely removed."

\* Received for publication March 31, 1908.

<sup>1</sup> Bulloch, *Lancet*, 1905, 2, p. 1603; Bulloch and Western, *Proc. Royal Soc.*, 1906, B., 77, p. 531.

Simon and his coworkers<sup>1</sup> were unable to obtain any evidence of specificity on the part of bacteriopsonins in normal serum; in their absorption experiments with *Staph. citreus* and *B. coli* the serum (pig) lost its opsonic power to the same extent for both organisms no matter which one was used to take up the opsonin. Potter, Dittman, and Bradley<sup>2</sup> obtained similar results. It must be mentioned, however, that in these experiments the absorption in no case was carried so far as to remove completely all the opsonin for the organism in question and that on this account the results, strictly speaking, may not be regarded as conclusive.

York and Smith<sup>3</sup> occasionally obtained complete suppression of the opsonic power of normal serum upon many organisms by the repeated addition of one.

Russell<sup>4</sup> concludes that the opsonins in normal sera are common opsonins since they can be removed by saturation with any one of a considerable number of bacteria. I may point out, however, that this conclusion does not seem warranted in a strict sense because Russell was not able by means of saturation of serum with staphylococci to remove completely the opsonin for *B. pyocyaneus*, the serum after saturation still causing more than twice the amount of phagocytosis as that obtained in the control experiments without any serum.

Axamit and Tsuda<sup>5</sup> report that after absorption of guinea-pig serum with *Staphylococcus*, *B. subtilis*, and *B. dysenteriae* there resulted in each case reduction in the opsonic power of the serum, but the reduction seemed in no instance specific so far as any of the three bacteria employed were concerned. The results are, however, not conclusive, in the first place because the absorption was not carried so far as to remove completely all the opsonin in the serum for the bacterium used, secondly because numerical methods were not employed in determining the results, and thirdly because the possibility of spontaneous phagocytosis did not receive adequate recognition.

Muir and Martin's<sup>6</sup> careful experiments brought out that in the

<sup>1</sup> Simon, Lamar, and Bispham, *Jour. Exp. Med.*, 1906, 8, p. 651; Simon, *ibid.*, 1907, 9, p. 487.

<sup>2</sup> *Jour. Am. Med. Assoc.*, 1906, 47, p. 1722.

<sup>3</sup> *Biochem. Jour.*, 1906, 2, p. 74.

<sup>4</sup> *Johns Hopkins Hosp. Bull.*, 1907, 28, p. 252.

<sup>5</sup> *Wien. kl. Wchnschr.*, 1907, 20, p. 1045.

<sup>6</sup> *Proc. Royal Soc.*, 1907, B., 79, p. 187.

case of a normal unheated serum every bacterium tested (*Staph. aureus*, *B. coli*, *V. metchnikovi*, *B. tuberculosis*, *B. pyocyaneus*) "absorbs large quantities of opsonin when the staphylococcus is used as the test for opsonic action. At the same time the staphylococcus appears to remove this opsonin more quickly than any of the others." Even after treating the serum twice there remained a difference as regards absorbing power in favor of *Staph. aureus*. Levaditi and Inmann<sup>1</sup> state that *B. typhosus*, *Staphylococcus* and *B. dysenteriae* each remove the opsonin for all three from rabbit serum, but their figures show that considerable opsonin remains for the staphylococcus after treatment of serum with typhoid bacilli.

Klien<sup>2</sup> found the opsonin for typhoid bacilli in the serum of rabbits immunized with typhoid bacilli to be quite specific, but that the typho-opsonin of normal rabbit serum could be absorbed completely by other bacteria (staphylococci) and for this reason he concluded that it must be largely non-specific. It is possible, however, that in this case the typho-opsonin which does not seem to be abundant in normal rabbit serum was removed as the result of a non-specific adsorption. This consideration is of course applicable to other results as well.

The fact that opsonins in normal serum, like complements and ferments, are removed from serum, wholly or in part, by various more or less finely divided organic and inorganic substances has been pointed out by a number of observers. Thus Simon and his co-workers<sup>3</sup> showed that opsonins are removed by flour, albumen, charcoal, chalk, etc. Neufeld and Hüne,<sup>4</sup> Levaditi and Inmann,<sup>5</sup> Levaditi and Kössler,<sup>6</sup> and Muir and Martin<sup>7</sup> have shown that opsonins are removed from normal serum by yeast and cellular debris, by the precipitate which forms on adding to a serum specific antiserum, and by red corpuscles or bacteria treated with immune body. Hence it has been suggested that the opsonins in normal serum are complements or alexins, i. e., amboceptor and complement.

<sup>1</sup> *Comp. rend. Soc. de Biol.*, 1907, 62, p. 683.

<sup>2</sup> *Johns Hopkins Hosp. Bull.*, 1907, 18, p. 245.

<sup>3</sup> *Jour. Exp. Med.*, 1906, 8, p. 651.

<sup>4</sup> *Arb. a. d. kais. Gesundh.*, 1907, 35, p. 1.

<sup>5</sup> *Comp. rend. Soc. de Biol.*, 1907, 62, pp. 685, 725.

<sup>6</sup> *Ibid.*, p. 685.

<sup>7</sup> *Proc. Royal Soc.*, 1907, 79, p. 187.

Now the absorption of opsonins by finely divided substances in no way necessarily signifies that normal opsonins are non-specific and, as I have indicated, it may be that the more or less complete removal of opsonin in some absorption experiments is due in some measure at least to physical adsorption. This possibility seems to have been overlooked in the investigations cited.

In the meantime it has been shown conclusively by Dean,<sup>1</sup> Neufeld and Bickel,<sup>2</sup> Levaditi and Inmann,<sup>3</sup> Cowie and Chapin,<sup>4</sup> that serum, speaking in general, owes its opsonic power to a thermostable element the activity of which is greatly increased by a coexistent thermolabile complement-like substance. This is true of normal as well as of immune serum; in the immune serum, however, the thermostable element appears to be increased enormously. This demonstration clears up certain obscure points in regard to the effect of heat upon opsonic serum at the same time as it makes it quite probable that the general loss of opsonic power by normal serum subjected to absorption experiments observed by some may be owing to the removal of the thermolabile substance or group of substances, whereas various thermostable opsonins may remain without being detected by the usual methods because present in comparatively small amounts. This suggestion applies also to the interpretation of the antiopsonic action upon normal serum of various procedures that remove or neutralize complements in general, e. g., complement deviation, treatment of serum with certain salts, inert materials, etc.

There are then good reasons against accepting as final the conclusion that opsonins in normal serum are non-specific so long as it is based solely upon the results of the experiments referred to in the foregoing. This reservation is indicated so much the more as other results are reported which corroborate the original conclusion of Bulloch and Western that normal serum contains specific opsonic substances, namely the results obtained by MacDonald and by Rosenow.

MacDonald<sup>5</sup> investigated the specificity of opsonins in human

<sup>1</sup> *Proc. Royal Soc., B.*, 1905, 76, p. 350; also 1907, 79, p. 350; *Brit. Med. Jour.*, 1907, 2, p. 1409.

<sup>2</sup> *Arch. a. d. kais. Gesundheit*, 1907, 27, p. 310.

<sup>3</sup> *Comp. rend. Soc. de Biol.*, 1907, 62, p. 683.

<sup>4</sup> *Jour. Med. Research*, 1907, 17, pp. 37, 95, 213.

<sup>5</sup> *Aberdeen University Studies*, 1906, 21, p. 323.



serum by the usual absorption method: serum was digested for 15 minutes at 37° C. with a definite quantity of thick pneumococcal suspension in normal salt solution and the same amount of salt solution was added to the control. The cocci were then removed by centrifugalization and the supernatant fluid was compared as to opsonic power with the control, the result showing that while the supernatant fluid had lost almost all the opsonin for pneumococci it had lost but very little opsonin for staphylococci. The remainder of the supernatant fluid was now divided into two equal parts and a quantity of thick staphylococcus suspension was added to one part while the same amount of salt solution was added to the other. After 15 minutes at 37° C. the cocci were removed by the centrifuge and the supernatant fluid was now found to have lost almost all its opsonin (87 per cent) for staphylococci but only 24 per cent of the opsonin for tubercle bacilli. From these results MacDonald concludes that there is an opsonin specific for each organism in human serum and that the changes in the opsonic content in a particular infection concerns only the opsonin for the infecting agent.

Rosenow's<sup>1</sup> experiments on this point gave even more striking results in the favor of specificity of opsonins in normal human serum. He took up the 24-hour surface growth of several large, plain agar slants (approximately 250 sq. cm.) of the bacteria used with 2.5 to 3 c.c. normal human serum. The mixtures were incubated from 3 to 12 hours or placed at room temperature for 24 hours and then at 37° C. for two additional hours. The heated serum, after dilution with an equal quantity of salt solution, was now filtered. The opsonic content of this serum was then compared with that of untreated serum which was kept under the same conditions of temperature and diluted to the same extent. The mixtures for determining the relative degrees of phagocytosis consisted of equal parts of washed normal blood, serum and salt solution, and bacterial suspension. The results showed that non-virulent pneumococci deopsonized human serum completely, so far as strains of avirulent pneumococci were concerned; at the same time they absorbed some but by no means all of the opsonin for streptococci, staphylococci, and tubercle bacilli. Streptococci and staphylococci, on the other hand, absorbed completely the opsonin

<sup>1</sup> *Jour. Infect. Dis.*, 1907, 4, p. 285.

for each respectively, but not nearly all the opsonins for the other bacteria.

The question of the specificity of the hemopsonins in normal serum has not received special attention and inasmuch as they lend themselves nicely to the experimental methods necessary to investigate such questions it seemed to me that it would be worth the while to subject them to closer study. For the study of the specificity of opsonic substances in apparently normal serum the hemopsonins may be considered as even more advantageous than the bacteriopsonins, because so far as we know they are much less likely to be influenced by actual immunizing processes that may be set in motion by slight and perhaps unrecognized infections. In other words, the normal hemopsonic substances are less likely to become mixed with immune hemopsonins than are the normal opsonic substances for bacteria to become mixed with immune bacteriopsonins concerning the specificity of which there is now no doubt.

In the first place, I wish to cite observations that indicate that hemopsonins occur which are distinct from opsonins for bacteria. In connection with some other experiments it was observed that the serum of a certain scarlet fever patient when mixed with the washed blood cream of a certain normal individual caused marked erythrocytic phagocytosis, from 14 to 50 per cent of the leucocytes at different times taking up red corpuscles. On treating the serum with an excess of corpuscles this hemopsonin disappeared completely from the serum which however retained its full normal opsonic value for gonococci. On heating the serum to 56° for 30 minutes both opsonins appeared to be completely destroyed, so far as indicated by the results of ordinary phagocytosis experiments.

Rabbit serum frequently contains opsonin for human corpuscles as shown by the taking up of the corpuscles by human leucocytes in the presence of rabbit serum. Removal of this opsonin by human corpuscles does not diminish the opsonic power of rabbit serum for bacteria.

Normal dog serum is strongly opsonic for anthrax bacilli and for rat corpuscles; absorption with large quantities of anthrax bacilli removes the opsonin for both bacilli and corpuscles, but absorption with sufficient corpuscles to remove practically all the hemopsonin

does not diminish at all perceptibly the opsonin for anthrax bacilli.

Many normal sera contain opsonins for various red corpuscles. Normal dog serum subjects the washed corpuscles of the rabbit, white rat, sheep, and goat to phagocytosis by the washed leucocytes of the dog, more particularly by the mononuclear cells in the exudate caused by injecting aleuronat into the pleural cavity. The fact that mononuclear cells take such prominent part in the phagocytosis of red corpuscles, and particularly of those of the rat, under the influence of normal dog serum is noteworthy. Guinea-pig corpuscles are also taken up, but because of the marked lytic effect upon them by dog serum they are not so suitable for phagocytosis experiments as the other corpuscles enumerated. On account of their great susceptibility to phagocytosis and their relatively marked resistance to lysis the corpuscles of the white rat are especially suitable for certain experiments in this line. The phagocytability of rat corpuscles is often so marked, however, that minute quantities of serum may cause more or less phagocytosis; hence in all experiments with washed leucocytes great care must be used to remove all traces of serum. Usually only a minute trace of spontaneous phagocytosis occurs.

I have made various absorption experiments with dog serum and the record of an experiment follows: In this instance 0.05 c.c. of fresh dog serum mixed with 0.1 c.c. of a 5 per cent suspension of washed corpuscles and 0.1 c.c. of a suspension of dog leucocytes (washed exudate), the whole being brought up to 0.3 c.c. by means of NaCl solution after one hour at 37° C., gave the following percentage in each case of mononuclear cells engaged in phagocytosis, these being practically the only kind of cell taking part in the phagocytosis so far as could be determined and the only cell counted in the experiments now reported:

Rabbit corpuscles.....	18
Rat                   ".....	60
Sheep               ".....	30
Goat                ".....	36

To 1.5 c.c. of a 20 per cent suspension of each of the four kinds of corpuscles named, carefully washed, were now added 0.25 c.c. of dog serum; after incubation at 37° C. for one hour the tubes were

centrifugated, the supernatant fluid very carefully removed, and in each instance one-fifth thereof ( $=0.05$  serum) added to  $0.1$  c.c. of a 5 per cent suspension of each of the corpuscles; the phagocytic mixtures were completed by adding to all  $0.1$  c.c. of leucocytic suspension and bringing the total quantity to the same point by adding NaCl solution when necessary. After one hour at  $37^{\circ}\text{C}$ . smears were prepared. The result is given in Table 1 the figures in which give the percentage of phagocytic mononuclear leucocytes.

TABLE 1.  
THE HEMOPSONIC PROPERTIES OF NORMAL DOG SERUM AFTER TREATMENT WITH VARIOUS CORPUSCLES.

DOG SERUM	CORPUSCLES			
	Rabbit	Rat	Sheep	Goat
Normal.....	18	60	30	36
Treated with rabbit corpuscles.....	0	60	26	6
Treated with rat corpuscles.....	18	30	40	35
Treated with sheep corpuscles.....	14	50	0	4
Treated with goat corpuscles.....	14	50	20	4

When normal dog serum is heated to  $58^{\circ}\text{C}$ . for 20 minutes its hemopsonic power is greatly diminished, as shown by the results of the following experiment in which each mixture contained  $0.1$  c.c. of a 5 per cent suspension of washed corpuscles,  $0.1$  c.c. of suspension of dog leucocytes (exudate) carefully washed,  $0.025$  of serum, and  $0.075$  of NaCl solution. The figures give the percentages of mononuclears engaged in phagocytosis.

TABLE 2.  
THE EFFECT UPON ITS HEMOPSONIC POWER OF HEATING DOG SERUM TO  $58^{\circ}\text{C}$ . FOR 20 MINUTES.

Corpuscles	Unheated Serum	Heated Serum
Rabbit.....	20	4
Rat.....	56	12
Sheep.....	6	3
Goat.....	16	6

Heated dog serum may be "activated" by the addition of minute quantities of normal fresh serum, that is, on mixing proper quantities of heated serum with dilutions of fresh serum greater phagocytosis results than the sum produced by the two kinds of sera acting independently (Table 3):

TABLE 3.  
ACTIVATION OF HEATED DOG SERUM.

Heated Serum	Unheated Serum	Phagocytosis
.025	....	15
.025	.006	25
.025	.0015	30
.025	.0004	22
....	.006	0
....	.0015	0
....	.0004	0

Each mixture in Table 3 contained 0.1 c.c. of 5 per cent suspension of rat corpuscles, 0.1 c.c. of suspension of dog leucocytes (washed exudate), both these elements having been carefully washed, the amount of serum indicated in the table with sufficient NaCl solution to make the total quantity 0.4 c.c. in each case. The figures show the percentage of phagocytic mononuclears.

In the absorption experiment the results of which are given in Table 1, the serum contained the thermostable opsonic substance as well as the adjuvant thermolabile element. The conditions consequently are complicated and in order to simplify matters similar experiments have been made with serum heated to 58° C. for 20 minutes. Table 4 gives the results of an experiment in which equal quantities of rat, goat, and sheep corpuscles were mixed in each case with 0.35 c.c. of heated serum (dog) and placed at 37° C. for one hour when the corpuscles were removed by means of the centrifuge; the usual phagocytic mixtures were then made so that each one contained 0.07 c.c. of the serum in question.

TABLE 4.  
THE HEMOPSONIC PROPERTIES OF HEATED DOG SERUM AFTER TREATMENT WITH VARIOUS CORPUSCLES.

DOG SERUM (Heated at 58° C. for 20 minutes)	CORPUSCLES		
	Rat	Sheep	Goat
Untreated.....	70	20	20
Treated with rat corpuscles.....	5	4	5
Treated with sheep corpuscles.....	36	2	2
Treated with goat corpuscles.....	70	9	0

Without going into any discussion of details at this time, it may suffice to say that the results of the absorption experiments here given, those with the unheated as well as those with the heated serum, seem to me to indicate that the hemopsonic action of normal dog serum

is dependent upon more than one substance, because excess of each kind of corpuscle removes most completely the opsonic substance or substances that seem peculiar to itself.

Of other examples of apparent specificity of hemopsonins in normal serum as indicated by the results of absorption experiments I may mention the removal by rabbit corpuscles from guinea-pig serum and goat serum of the substances that render them phagocyttable by dog leucocytes, whereas the opsonic substances for rat corpuscles are left behind at the same time as the reverse is true of the action of rat corpuscles upon these sera.

In the foregoing I pointed out that the results obtained by Bulloch and Western, MacDonald, and Rosenow certainly indicate that normal human serum contains opsonic substances with pronounced specific affinities for certain bacteria. The conclusion that such is the case receives strong support from the results of observations on the opsonic index under certain conditions.

Recent investigations in this institute have shown that in the early stages of many acute human infections as well as immediately after the inoculation of killed bacteria into normal animals and normal human beings there usually occurs a distinct and sometimes pronounced fall below the normal in the opsonic index for the bacterium causing the infection or used in the inoculation, whereas the index for other bacteria remains unaffected. Thus Dr. Tunncliffe's<sup>1</sup> observations show that in the early stages of scarlet fever and of erysipelas<sup>2</sup> the streptococco-opsonic index is below normal, whereas the index for other bacteria is within normal limits. Numerous other observations of the same import might be cited; two illustrations are given in Chart 1.

As is well known marked variations in the specific opsonic index also occur in chronic infections. Now these variations cannot be variations in newly formed immune opsonins only because the index often is subnormal.

These facts indicate that normal opsonins are specific or, speaking more broadly, that infectious agents affect the mechanism of opsonification in a specific manner. If normal human blood contains only one common opsonic substance, then one would expect

<sup>1</sup> *Jour. Infect. Dis.*, 1907, 4, p. 304

<sup>2</sup> See Chart 3, p. 270 of this number.

the fall in the index following a given infection to be more general in its scope and not limited so sharply to the index for the infecting organism. Whether the so-called "negative phase" of the opsonic index is the expression of a diminution in the complement-like element or in the thermostable opsonic element in the blood does not seem to have been studied. Muir and Martin<sup>1</sup> suggest that the fall may be owing to a diminution in the "complement-like opsonin," but the evidence at hand furnishes strong indications that fall as well as rise in the index result from fluctuations in the thermostable substance. Thus the observation by Dean that sensitized cocci do not absorb opsonin from immune or from normal serum speaks in favor of the identity of normal and immune opsonins. Clark and Simonds<sup>2</sup> show that normal human and normal rabbit sera after being heated to 56-8° C. for 20 minutes possess the power

to promote phagocytosis of typhoid and paratyphoid bacilli by washed human leucocytes. On comparing this power of the serum of normal rabbits with that of the serum of rabbits injected with killed typhoid and paratyphoid bacilli a distinct fall below normal was noted soon after the first injections. In connection with this question I may point out that when human serum heated to 60° C. for 10 minutes

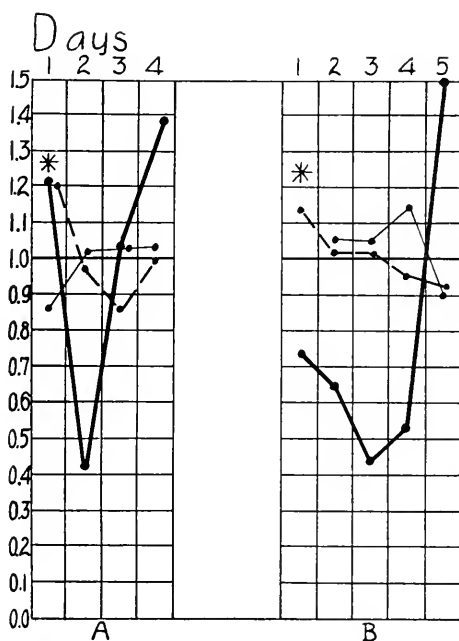


CHART 1.—THE SPECIFICITY OF THE "NEGATIVE PHASE" OF THE OPSONIC INDEX.

A=Opsonic curves of patient inoculated with dead streptococci at \*; B=Opsonic curves in case of rabbit inoculated with dead streptococci at \*; heavy line=streptococcus index; broken line=pneumococcus index; fine line=staphylococcus index.

<sup>1</sup> *Proc. Royal Soc.*, 1907, B, 79, p. 181.

<sup>2</sup> *Jour. Infect. Dis.*, 1908, 5, p. 1.

is mixed with enough staphylococci to make a thick paste, incubated at 37° C. for 45 minutes, and then completely freed from cocci by centrifugation at high speed, it will be found by suitable activation experiments<sup>1</sup> to have lost its thermostable opsonic substance so far as staphylococci are concerned whereas the thermostable opsonic substance peculiar for pneumococci remains in the same proportion as in normal serum; the reverse takes place when heated serum is treated with pneumococci—the thermostable pneumococco-opsonic element is removed while the thermostable staphylococco-opsonic element persists (Table 5).

TABLE 5.

THE EFFECTS UPON HEATED HUMAN SERUM OF ABSORPTION WITH STAPHYLOCOCCI AND PNEUMOCOCCI.

SERUM	PHAGOCYTOSIS (The figures give average number of cocci taken up per leucocyte)	
	Staphylococci	Pneumococci
Staphylococcus serum+normal serum 1:10.....	1.1	2.0
Pneumococcus serum+normal serum 1:10.....	2.5	1.0
Untreated heated serum+normal serum 1:10.....	3	1.6
Salt solution only.....	1.1	0.9

Each mixture contained one volume of heated serum, treated or untreated as the case might be; one volume of normal serum diluted 1 to 10; one volume of suspension of washed blood cream; and one volume of suspension of cocci; in the control mixture the sera were replaced by two volumes of NaCl solution.

This result harmonizes well with the view that the specific fall in the index is due to diminution of the thermostable opsonic element and in Chart 2 I show that the opsonic index of the serum of a dog after intravenous injection with rat corpuscles presents the same curve when determined with heated and unheated serum; furthermore, that the fall following the injection appears specific in this case also.

I am well aware that Landsteiner<sup>2</sup> and others question the correctness of the interpretation that the results of absorption experiments indicate the existence of specific agglutinins and lysins in normal serum. The conclusion, however, from absorption experiments that normal serum contains opsonic substances with specific affinities

<sup>1</sup> See article by H. E. Eggers, p. 263 of this issue.

<sup>2</sup> *Ztschr. f. Hyg.*, 1907, 58, p. 213.



receives direct support from the specificity of the fall in the opsonic index under certain conditions.

#### SUMMARY.

The results of the absorption experiments by Bulloch and Western, MacDonald, and Rose now indicate that normal human serum may contain several bacteriopsonins with more or less well-marked specific affinities. The results of further absorption experiments indicate that the bacteriopsonins and hemopsonins in human, rabbit, and dog serum may be distinct substances, at least in part, and that normal serum may contain several more or less distinctly specific opsonins for alien red corpuscles. The results of absorption experiments with heated serum and the demonstration of a specific negative phase in the opsonic index when determined with heated serum point directly to the thermostable opsonic elements in normal serum as the carriers of the specific affinity, but further studies are required upon the complement-like substances of serum. The claim that normal serum contains specific opsonic substances receives strong support from the observation that soon after infection or inoculation there may occur a depression in the opsonic index which is specific for the infecting or inoculated microbe. This specificity of the negative phase of the opsonic index appears to hold true also when alien red corpuscles are used as antigens. The presence in normal serum of several more or less specific opsonic substances and the specific depression of the opsonic index on the entrance into the body of infectious or antigenic material accord well with the view

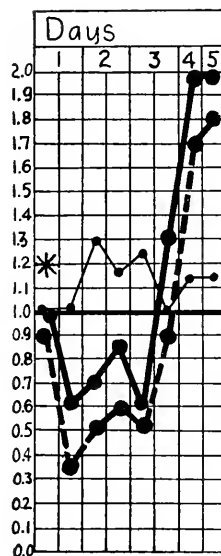


CHART 2.—THE HEMOPSONIC INDEX OF THE SERUM OF DOG INJECTED WITH CORPUSCLES OF WHITE RAT.

The dog was four months old and weighed seven pounds. At \* it received 0 c.c. of 10 per cent suspension of washed corpuscles.

Heavy solid line=index obtained with unheated serum each mixture containing 0.1 c.c. of 5 per cent suspension of rat corpuscles, 0.1 c.c. of leucocytic suspension and 0.01 of serum+0.09 of NaCl solution; broken line=index obtained with serum heated to 58° C. for 30 minutes, the mixtures in this case containing 0.02 of serum; fine line=index for goat corpuscles with unheated serum. In each case the index means the relation of the percentage of phagocytic mononuclears in the mixture made with the serum of the injected dog to the percentage of phagocytic mononuclears in the mixture made with normal dog serum.

expressed by Wright on the basis of Ehrlich's theory of antibody formation, namely "that in immunization we never start from a base of non-resistance, but call into existence an increased quantity of such chemical agents as already are performed in the body." Finally it may be pointed out that specific depression of the opsonic index in certain infections probably is the result of infection rather than, as suggested by some, an antecedent condition favoring infection.

# ON THE INTERACTION OF THERMOLABILE AND THERMOSTABLE OPSONIC SUBSTANCES IN NORMAL SERUM.\*

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THAT opsonins normally present in serum were destroyed by heating to 60° for 10 minutes was ascertained by Wright and Douglas.<sup>1</sup> Hektoen and Ruediger<sup>2</sup> found that "opsonins are thermolabile substances of a constitution analogous to that of toxins and complements in that they seem to have two groups, haptophore and opsoniferous." Other observers, among them Levaditi,<sup>3</sup> Savtschenko,<sup>4</sup> Taraskevitch,<sup>5</sup> and Neufeld<sup>6</sup> and his coworkers, have found that opsonic substances in immune sera are largely thermostable, in that respect apparently differing from the sensitizing bodies in normal serum. Muir and Martin<sup>7</sup> were able to establish other differences in behavior between normal and immune opsonic substances and came to the conclusion that the thermolabile opsonin of normal serum and the thermostable opsonin of immune serum are distinct classes of substances, showing different combining relationships as well as different heat resistances.

In 1907 Dean<sup>8</sup> pointed out that on mixing appropriate dilutions of a heated immune serum with dilutions of normal fresh serum, phagocytic indices were obtained greater than resulted from the sum of the two acting separately. Later Cowie and Chapin<sup>9</sup> were able to show a similar phenomenon in the case of heated and diluted normal human serum with respect to phagocytosis of staphylococci. In

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<sup>1</sup> *Proc. Royal Soc.*, 1903, 72, p. 357; 1904, 73, p. 128.

<sup>2</sup> *Jour. Infect. Dis.*, 1905, 2, p. 128.

<sup>3</sup> *Ann. de l'Inst. Past.*, 1901, 15, p. 904.

<sup>4</sup> *Ibid.*, 1902, 16, p. 106.

<sup>5</sup> *Ibid.*, 1902, 16, p. 127.

<sup>6</sup> Neufeld and Töpler, *Centralbl. f. Bakt.*, 1907, 38, p. 456; Hüne, *Arb. a. d. kais. Gesundh.*, 1907, 25, p. 164.

<sup>7</sup> *Proc. Royal Soc.*, 1907, 79, p. 187.

<sup>8</sup> *Proc. Royal Soc.*, 1907, 79, p. 399; see also *ibid.*, 1905, 76, p. 506.

<sup>9</sup> *Jour. Med. Res.*, 1907, 17, pp. 95, 213.

the same year Neufeld and Bickel<sup>1</sup> found that "sublytic" amounts of serum containing hemolytic amboceptor, in the presence of minute quantities of normal serum, could so act on erythrocytes as to induce active phagocytosis.

The results in this article are based on observations along similar lines upon normal serum exclusively.

Except where otherwise indicated, an avirulent strain of pneumococcus was used throughout. It was kept up on blood-agar, suspensions for immediate use being prepared by growing for 24 hours in glucose-free broth; this method, worked out by Dr. Rosenow in this laboratory, has been found to yield very satisfactory suspensions. The technic otherwise was essentially that of Wright and Douglas.

At the outset, using only one specimen of human serum (Serum "E") and working with pneumococcus, I was unable to reproduce the results of the previous investigators. The following table (Table 1) serves to illustrate the character of the results repeatedly obtained with this serum. Diluted serum is normal serum ("E"), diluted 10 times in NaCl solution. Heated serum is "E" serum heated to 60° for 10 minutes. Leucocytes are human blood cream, washed. The figures give the average number of bacteria taken up per leucocyte, 50 leucocytes being counted; 100 if individual counts showed much variation.

TABLE 1.

Dil. serum + leucocytes + bact. susp. + NaCl sol.	= 1.9.
H't " + " + " + " + "	" = 1.0
" " + " + " + " + dil. serum	= 2.6
Nor. " + " + " + " + NaCl sol.	= 5.2

I then tried to substitute diluted guinea-pig serum for the diluted human serum. All factors are as in preceding experiments, except that dilute serum is guinea-pig serum diluted 10 times. Table 2 shows the results so obtained.

TABLE 2.

Heated serum + leucocytes + bact. susp. + NaCl sol.	= 1.4
G. P. ser. dil. + " + " + " + "	" = 1.1
" " + " + " + " + h'd serum	= 2.8
Nor. human ser. + " + " + " + NaCl sol.	= 6.4
Nor. G. P. " + " + " + " + "	" = 3.8

We see that the use of guinea-pig serum diluted, in place of diluted human serum, did not promote phagocytosis by the heated serum.

<sup>1</sup> *Arch. a. d. kais. Gesundh.*, 1907, 27, 310.

Experiments were also instituted to determine whether or not variations in the dilution of the suspension of organisms could account for the results. Full, half, and quarter strength dilutions of the suspended organisms were used, with negative results. Inasmuch as the explanation was finally found in another direction, insertion of the results here is scarcely necessary.

At this point another human serum ("G") was used, all other factors being as in Table 1; the results obtained with this are given in Table 3.

TABLE 3.

"G" serum heated,	leucocytes,	bact. susp.,	NaCl sol.	=0.7
" " diluted,	" "	" "	" "	=0.7
" " heated,	" "	" "	"G" ser. dil.	=2.6
" " normal,	" "	" "	NaCl sol.	=3.0

With this serum results apparently corroborating those of previous investigators were obtained.

The next experiment (Table 4), was made in an effort to differentiate the particular factor that prevented the promotion of phagocytosis in the case of the first serum (Serum "E"). Sera "E" and "G" and corpuscles "E" were used, all other factors as before.

TABLE 4.

"E" ser.	h'd.	+ leucocytes	+ bact. susp.	+ NaCl sol.	=0.6
" "	dil.	+ "	+ "	" "	=0.4
" "	"	+ "	+ "	"E" ser. h'd.	=0.9
" "	nor.	+ "	+ "	+ NaCl sol.	=4.3
"G" "	h'd.	+ "	+ "	" "	=1.0
" "	dil.	+ "	+ "	" "	=0.5
" "	"	+ "	+ "	"G" ser. h'd.	=4.0
" "	nor.	+ "	+ "	+ NaCl sol.	=3.9
"E" "	h'd.	+ "	+ "	"G" ser. dil.	=0.7
"G" "	"	+ "	+ "	"E" "	=4.1

It is evident that all mixtures depending on "E" serum heated for phagocytosis show no so-called activation. Hence the cause of the negative results with "E" serum must be attributed to some peculiarity in the thermostable opsonic element in this serum.

Sera from different individuals were now tested to ascertain the relative frequency of this phenomenon of "inactivability" and in nine of twelve individuals results were obtained similar to those with serum "E."

To find whether or not "E" serum would act in the same way toward other organisms as toward pneumococcus, it was tested with

one strain each of *Strept. pyogenes* and *Staph. albus*. The results are shown in Table 5.

TABLE 5.

Mixtures						Phagocytosis	
						<i>Strept.</i>	<i>Staph.</i>
"E" ser.	h'd.	+ leucocytes	+ bact. susp.	+ NaCl sol.		=2.5	1.5
" "	dil.	+	"	+	"	=2.3	5.4
" "	"	+	"	+	"	=4.0	7.4
" "	nor.	+	"	+	"	=6.5	9.5
"G" "	h'd.	+	"	+	"	=3.1	1.5
" "	"	+	"	+	"	=7.7	8.4
" "	nor.	+	"	+	"	=9.5	11.3

From the results given, it is evident that the behavior of "E" serum toward streptococcus is like that toward pneumococcus. With staphylococcus, on the other hand, there is apparently some degree of reactivation, though not so great as in the case of serum "G."

From the work of Dean, Neufeld and Bickel, and Cowie and Chapin, it would appear that normal opsonin may consist of thermostable and thermolabile elements, similar to the amboceptor and complement constituting bacterial antibodies. Neufeld,<sup>1</sup> indeed, views the process of opsonification as one of incipient lysis, basing his view chiefly on the results observed by himself and Bickel. However, he notes that at times concentrated normal serum, even after heating, favors phagocytosis of certain organisms; this phenomenon he does not attempt to explain.

Levaditi,<sup>2</sup> as well as Muir and Martin,<sup>3</sup> hold the view that opsonins in normal sera are, at least for the greater part, complements. This view Levaditi bases on the following facts: (1) like complement, opsonin is removed from rabbit serum by various micro-organisms and by cellular débris; (2) the serum of rabbits, injected with guinea-pig serum, neutralizes both the complement and opsonin of guinea-pig serum, the neutralization being specific; (3) phosphorous poisoning reduces both complement and opsonin; (4) aqueous humor contains neither complement nor opsonin. None of these facts is incompatible with the view that the complement-like constituent of opsonin, and complement, are similar in behavior, and the apparent removal of opsonin results from removal of its complement-like constituent only.

<sup>1</sup> *Arch. d. kais. Gesundh.*, 1907, 27, p. 414.

<sup>2</sup> *Compt. rend. Soc. de Biol.*, 1907, 67, p. 683.

<sup>3</sup> *Brit. Med. Jour.*, 1906, 2, p. 1783; *Proc. Roy. Soc.*, 1907, 79, p. 187.

## SUMMARY.

The results of Dean and of Cowie and Chapin to the effect that diluted fresh normal serum may increase greatly the opsonic power of heated serum are confirmed. At the same time a large proportion (75 per cent) of the different sera tested were found to contain inactivable thermostable elements at least with respect to the pneumococcus. In the case of one serum the thermostable element proved inactivable with respect to pneumococcus and streptococcus and activable in a small degree with respect to staphylococcus.

## THE OPSONIC INDEX IN ERYSIPELAS.\*

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AN increase in the streptococcal opsonin during the course of erysipelas has been observed by G. F. Ruediger.<sup>1</sup> Two cases examined by me<sup>2</sup> showed a rise as the symptoms subsided. Schorer<sup>3</sup> found no constant change in the streptococco-opsonic index corresponding with desquamation and recovery in two cases in which several estimations were made. In neither case did he make daily examinations. A composite chart of 36 cases observed by Schorer shows that erysipelas causes an increase in opsonin which reaches its height about the third day of the disease and then falls gradually to normal. Two cases examined by him on the first day of the disease had indices of 0.6 and 0.3.

In order to ascertain whether the streptococco-opsonic index follows a definite course during an attack of erysipelas, I have made daily examinations in several cases. A typical *Strept. pyogenes* isolated from a case of otitis media in erysipelas was employed for the suspension. In the routine examinations the opsonic indices were estimated in the usual way and after incubation of the mixtures at 37° C. for 15 minutes.

Ten patients have been examined. Isolated examinations were made in five cases. Five patients, one during three attacks, were examined as a rule daily during the course of the disease. It appears from the results that the index corresponds with the clinical symptoms rather than with the day of the disease. Of eight cases examined during the early period of the attacks, six had indices below normal ranging from 0.2-0.7, one patient had an index of 0.83, and another of 1.1. In all of the patients examined later in the disease except in a fatal case (Chart 2), there was found to occur with the fall in temperatures and improvement in the symptoms a rise in the streptococcal index varying from 1.4 to 4.4 (Chart 1), the average highest point

\* Received for publication March 31, 1908.

<sup>1</sup> *Jour. Infect. Dis.*, 1907, 4, p. 304.

<sup>2</sup> *Jour. Amer. Med. Assoc.*, 1906, 46, p. 108.

<sup>3</sup> *Amer. Jour. Med. Sci.*, 1907, 134, p. 728.



being 2.0. This rise was followed by an abrupt fall to normal in from one to three days. The serum of the patient with two recurrences showed with the onset of each a subnormal index, followed by a rise as the symptoms subsided (Chart 3).

In one case the opsonic indices were determined with respect to staphylococcus, the pneumococcus, *Strept. viridans*, *Strept. mucosus*, and three strains of *Strept. pyogenes*. *Strept. mucosus* was isolated from the spinal canal in a case of meningitis. The pyogenic

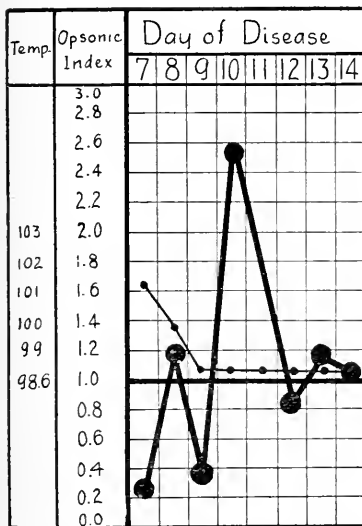


CHART 1.—Streptococco-opsonic index (heavy line) in erysipelas (man, 20 years) first attack.

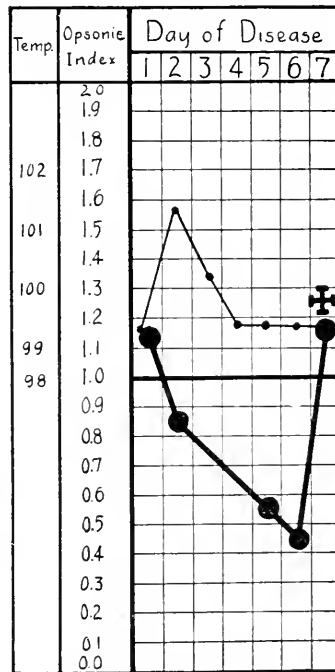


CHART 2.—Streptococco-opsonic index (heavy line) in fatal case of erysipelas (man, 40 years).

streptococci were isolated from the pus of inflammation of the middle ear in the course of an attack of erysipelas, from a bleb on the face of an erysipelas patient, and from the throat of a patient with acute articular rheumatism. Chart 3 shows that while the opsonic index varies as regards *Strept. pyogenes*, it remains within normal limits with respect to the staphylococcus, the pneumococcus and *Strept. viridans*. Chart 4 demonstrates that the opsonic indices to the strains of *Strept. pyogenes* and the *Strept. mucosus* correspond closely.

On heating normal and immune sera at  $44^{\circ}$  to  $49^{\circ}$  C. for 20 minutes before the mixtures were prepared for incubation, opsonic indices were obtained such that while they differ in height closely correspond in their course with those obtained in the usual way (Chart 5).

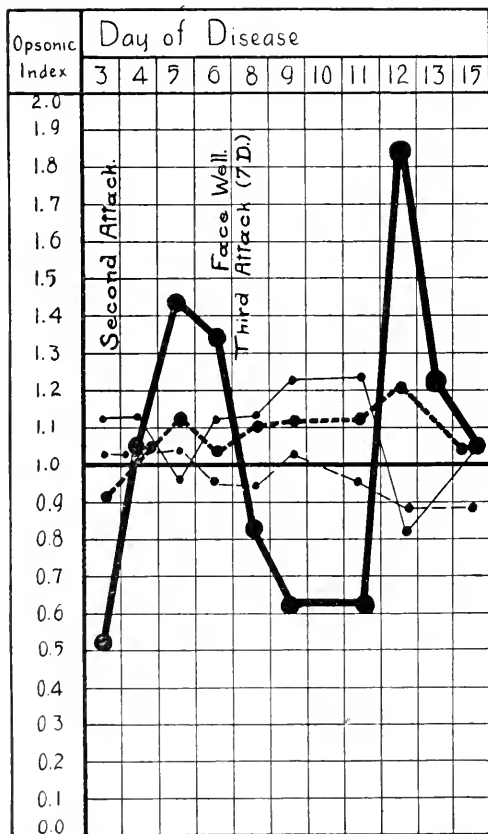


CHART 3.—Comparison of the opsonic index obtained in recurrent facial erysipelas<sup>2</sup> (man, 20 years), with respect to *Strept. pyogenes*, *Staphylococcus*, *Pneumococcus*, and *Strept. viridans*. Solid fine line=opsonic index to *Staphylococcus*. Broken heavy line=opsonic index to *Pneumococcus*. Broken fine line=opsonic index to *Strep. viridans*. Solid heavy line=opsonic index to *Strept. pyogenes*.

The comparative opsonic power of the serum to streptococcus was determined also by diluting normal and immune serum to the point of opsonic extinction as suggested by Simon<sup>1</sup> and practiced by Klien<sup>2</sup> with respect to typhoid bacilli, and myself<sup>3</sup> with diphtheria bacilli. The curves (Chart 5) representing the opsonic power estimated in this

<sup>1</sup> *Jour. Exp. Med.*, 1906, 8, p. 651; 1907, 9, p. 487.

<sup>3</sup> *Jour. Infect. Dis.*, 1905, 5, p. 14.

<sup>2</sup> *Johns Hopkins Hosp. Bull.*, 1907, 18, p. 245.

way correspond fairly closely with the curves of the opsonic indices estimated by the Wright method. In the case of indices of about 1.3 as estimated in the usual way no increase in the opsonic power is evidenced by the dilution method, but when the ordinary indices reach 1.8 or higher the results of the two methods correspond closely.

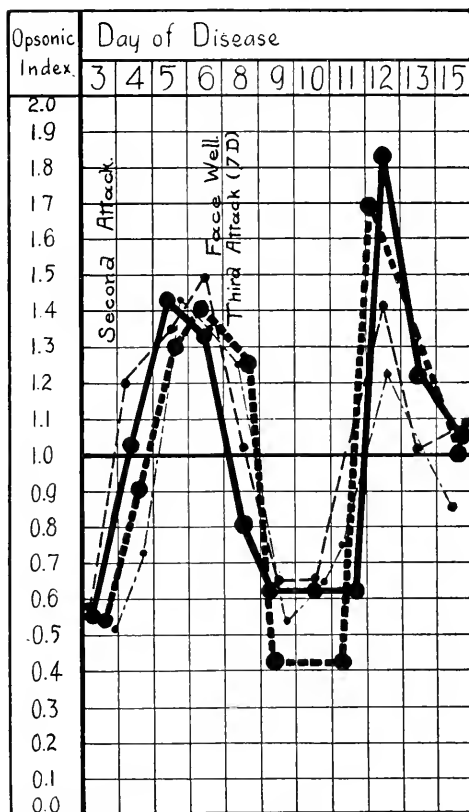


CHART 4.—Comparison of the opsonic index obtained in recurrent facial erysipelas (man, 20 years), with respect to three strains of *Strept. pyogenes* and a *Strept. mucosus*. Broken heavy line=opsonic index to *Strept. mucosus*.

#### CONCLUSIONS.

In erysipelas the opsonic index to *Strept. pyogenes* is generally below normal during the acute stage of the disease. As the symptoms subside and the temperature falls to normal the index rises considerably, returning to normal in from one to three days.

While these changes occur in the opsonic index with respect to *Strept. mucosus* and various strains of *Strept. pyogenes*, the index remains normal to the staphylococcus, the pneumococcus, and *Strept. viridans*.

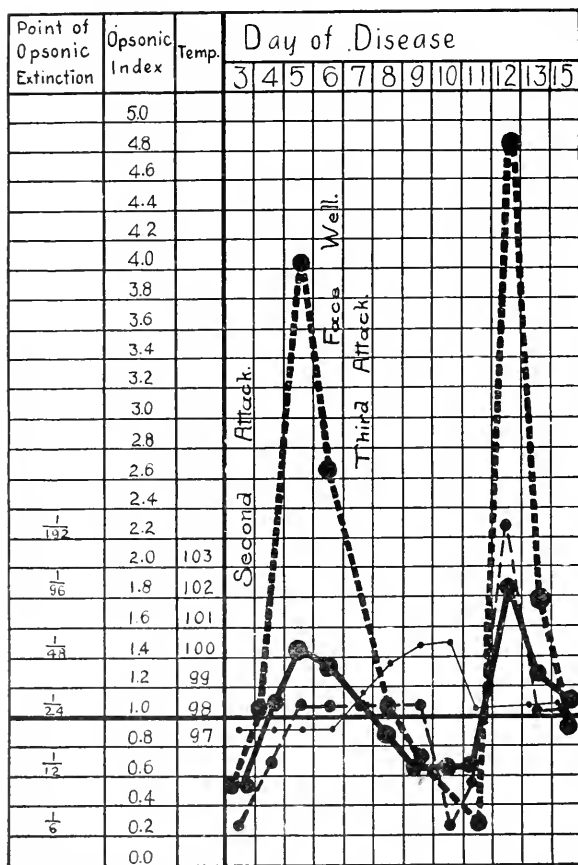


CHART 5.—Comparison of streptococcal opsonic indices in recurrent facial erysipelas (man, 20 years), obtained (a) with sera heated to 44–49° C. for 20 minutes (heavy broken line), (b) with unheated sera (heavy solid line), and (c) by dilution of sera to the point of opsonic extinction (fine broken line). Temperature=fine solid line.

The streptococcal indices obtained by first heating the serum at 44° to 49° C. for 20 minutes are higher but correspond closely in their course to those obtained by using unheated serum.

Curves obtained by estimating the opsonic power on diluting the serum to the point of opsonic extinction correspond fairly closely to those obtained by the Wright method and by the use of heated serum.

## ON THE PHAGOCYTABILITY OF PNEUMOCOCCI IN THE SPUTUM IN PNEUMONIA.\*

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PARK and Williams used the mass culture method<sup>1</sup> of inoculation into rabbits and white mice in order to determine the virulence of pneumococci isolated from sputum. The organisms so obtained from 87 per cent of pneumonic patients and from 69 per cent of healthy individuals were virulent when 4 c.c. were inoculated. When only 1 c.c. was inoculated 51 per cent of pneumonic patients and 31 per cent of healthy individuals showed virulent pneumococci. Buerger<sup>2</sup> obtained somewhat different results by injecting 24-hour pure cultures of pneumococci obtained from sputum into white mice. He found 79 per cent of virulent organisms in normal cases and 77 per cent in pneumonia cases. The results of these two independent researches show that the difference between the normal and pneumonic sputum in content of virulent pneumococci is at most only slight. Of further interest because showing a high percentage of virulent pneumococci in normal sputum is the work of Frost, Divine, and Reineking<sup>3</sup> who found that 90 per cent of rabbits died within 24 hours after the injection of 2 c.c. of saliva of healthy persons intraperitoneally in the spring months; 73 per cent in the winter; and 42 per cent in the autumn. Only those animals were considered which showed a pneumococcus septicaemia.

The object of the present work was to study further the virulence of pneumococci in pneumonic sputum, using susceptibility to phagocytosis as the criterion. Rosenow<sup>4</sup> has shown that highly virulent pneumococci do not absorb opsonin in normal serum and resist

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<sup>1</sup>"The mass culture method consists in inoculating a mass of sputum to be tested into serum-broth (previously tested for ability to give abundant growth of pneumococci), placing at 36° C. for 24 hours, and inoculating a certain amount of the resulting culture subcutaneously into the animal chosen. The culture isolated from the heart's blood of the animal at autopsy is then tested for virulence in the same species of animal."—Park and Williams, *Jour. Exper. Med.*, 1905, 7, p. 403.

<sup>2</sup>*Jour. Exper. Med.*, 1905, 7, p. 497.

<sup>3</sup>*Jour. Infect. Dis.*, 1905, Supplement No. 1, p. 208.

<sup>4</sup>*Jour. Infect. Dis.*, 1906, 3, p. 683.

phagocytosis; hence this method may be used for determining the virulence of single strains of organisms.

The series studied include 26 cases of lobar pneumonia, 16 before crisis, 4 on day of crisis, 5 after crisis, and 1 during lysis. They were chosen miscellaneous during February and March, 1907, from cases in hospitals. The duration of the disease varied from the second day after the chill to the fifth day after the crisis. In addition to these, two cases of bronchitis were studied and also two cases in which cultures were obtained post mortem from the tracheal and bronchial mucous membranes of individuals who died with lobar pneumonia.

In each case, except the two studied after death, the sputum was received in a sterile petri dish, washed in sterile broth, and immediately plated out in blood-agar; as soon as colonies appeared (always within 24 hours) subcultures upon blood-agar slants were made from six small green nonhemolyzing colonies.<sup>1</sup> On the following day opsonic experiments were made in the usual manner with normal human serum, human leucocytes, and a suspension of the bacteria in physiological salt solution. The mixtures were incubated for 30 minutes.

As a check on the identity of the organism each subculture was transferred to a tube of Hiss's inulin serum-water as modified by Ruediger.<sup>2</sup> This was particularly useful as a means of differentiating the pneumococcus from *Strept. viridans* and *Strept. mucosus*, two organisms described by Schottmüller which occur in the mouth and which also form slightly greenish colonies on blood-agar plates. If fermentation with acid production occurred in six days the organisms were considered to be pneumococci, and those organisms which failed to ferment inulin were left out of the final results.

In all 127 strains of organisms from pneumonic sputum and fulfilling these requirements were studied. No strain was allowed to grow

<sup>1</sup> See Schottmüller, *Munch. med. Wchnschr.*, 1903, 50, p. 849; Rosenow, *Jour. Infect. Dis.*, 1904, 1, p. 308.

<sup>2</sup> *Jour. Infect. Dis.*, 1906, 3, p. 755: "Dissolve 5 gr. of NaCl, 20 gr. of peptone (Witte), and 20 gr. of pure inulin in 1,000 c.c. of distilled water. Add 20 c.c. of a 5 per cent solution of pure litmus and tube, putting 2 c.c. of the mixture into each tube, and sterilize in the autoclav. After sterilization add (with a sterile pipette) 2 c.c. of sterile heated ascites fluid, or preferably heated beef serum, to each tube, and incubate for 24 hours before using. Great care must be observed not to use ascites fluid which contains fermentable carbohydrates. Each lot must be tested with organisms that are known to have great fermentative powers and if acid is produced it must not be used."

on artificial media for more than 72 hours before examination for fear of loss of virulence, although this danger is slight within a reasonable period, according to Rosenow<sup>1</sup> who in one case found a high degree of virulence preserved in growths upon blood agar after 163 days. Most of the strains were examined within 48 hours after collecting the sputum. Approximately uniform suspensions were used.

Of the total number of strains studied 119 were found to be easily susceptible to phagocytosis and only eight strains resisted phagocytosis or were taken up in very small numbers. In other words 93.7 per cent of organisms could be considered as avirulent, or only slightly virulent; and only 6.3 per cent of virulent organisms were found. The virulent organisms were obtained three hours before crisis, once during crisis and once four days after crisis.

In two cases of pneumonia several strains of pneumococci obtained from swab cultures of the tonsils were examined, cultures being taken at the same time from the sputum. No appreciable difference between the susceptibility to phagocytosis could be discovered between the organisms isolated from the tonsils and those from the sputum. Four strains were examined from two cases of acute bronchitis, and all were found to be easily susceptible to phagocytosis.

As a control of the reliability of estimating the virulence of pneumococci isolated from sputum by the susceptibility to phagocytosis two rabbits were injected intraperitoneally with 1 c.c. of a dense salt solution suspension of organisms from two different cases of pneumonia respectively. Both strains of organisms used were easily susceptible to phagocytosis and when injected into the animals had grown 48 hours upon blood-agar slants. One rabbit was unaffected except for a loss of 300 grams in weight. The other died after 28 days, and the pneumococcus was recovered in pure culture from the heart's blood. Therefore the virulence of the organisms must be assumed to have been of rather low grade.

Ten strains of pneumococci obtained post mortem from the tracheal and bronchial mucous membranes 12 hours after the death of a man from lobar pneumonia were uniformly readily susceptible to phagocytosis. Five cultures from the parenchyma of the lung were examined of which two were readily taken up by the leucocytes and

<sup>1</sup> *Jour. Infect. Dis.*, 1904, 1, p. 280.

three resisted phagocytosis. Five strains of pneumococci isolated from the pulmonary vein in the same case were all insusceptible to phagocytosis. Five strains isolated post mortem in another case from the tracheal and bronchial mucous membrane were all readily ingested by the leucocytes.

With a view to determining the possibility of increasing by symbiosis the virulence of organisms regarded as non-virulent two strains which were readily ingested by the leucocytes were grown together for 48 hours on blood-agar. At the expiration of this time they were still easily susceptible to phagocytosis. A subculture of one of these strains and of one obtained from the pulmonary vein post mortem were likewise grown together for 48 hours. Tests made with a suspension of the combined growth showed a complete resistance to phagocytosis. This result is in keeping with the finding of Rosenow<sup>1</sup> that an extract in salt solution of virulent pneumococci is capable of restoring a non-virulent strain to virulence and of rendering them insusceptible to phagocytosis. For it is possible that in this experiment the non-virulent organisms were made virulent by coming into contact with "virulin" from the strain which resisted phagocytosis. Of course other possibilities suggest themselves.

Assuming in the light of Rosenow's work that insusceptibility of pneumococci to phagocytosis may be considered as a criterion of virulence we see that by far the majority of pneumococci in the sputum of at least those cases of lobar pneumonia which I studied were non-virulent according to this standard. It is evident that no relation exists between the duration of pneumonia and the virulence, thus determined, of the organisms in the sputum. For as shown here, of the few strains that may be regarded as virulent some were obtained before crisis, one during crisis, and one as late as four days after crisis. These results differ radically from those of Park and Williams and the other authors I have cited, and this difference is no doubt owing to the difference in method.

The presence of so large a percentage of non-virulent pneumococci in pneumonic sputum, i. e., non-virulent because susceptible to phagocytosis, seems to me to be rather surprising because in practically all cases of lobar pneumonia there is a pneumococcemia of virulent

<sup>1</sup> *Jour. Infect. Dis.*, 1907, 4, p. 285.



organisms, i. e., those insusceptible to the action of normal opsonin (Rosenow). Naturally one would expect a considerable number of such organisms in the sputum of pneumonic patients. The strains isolated from the pulmonary vein in a case of pneumonia were uniformly insusceptible to phagocytosis while strains from the tracheal and bronchial membranes of the same case were readily phagocyttable. It lies near at hand to assume that perhaps only virulent organisms gain access to the blood stream. Another possible explanation is that organisms from the blood stream are excreted through the respiratory mucous membranes after being so changed that they lose the virulent property by virtue of which they resist the action of opsonin. In this connection the observations of Buerger and Ryttenberg<sup>1</sup> are of great interest. These observers found that pneumococci in the blood of a patient with a general infection with this organism may differ in cultural characteristics from those obtained from the metastatic foci, showing that the medium has a pronounced effect upon the biological properties of the pneumococcus even when it is growing in the living body. A third possibility also suggests itself, namely that mucus and saliva may possess properties that render virulent pneumococci phagocyttable if sufficient time is given. In the latter case it could be assumed that the virulent organisms encountered in the sputum are the last ones to come into contact with the mucus and saliva. With this last idea in view the following experiment was made: A strain of virulent pneumococci obtained by blood culture from a case of lobar pneumonia was suspended in salt solution to which an equal portion of filtered normal saliva was added. Blood-agar plates were made  $\frac{1}{2}$  hour,  $3\frac{1}{2}$  hours, and  $17\frac{1}{2}$  hours later. The suspensions were kept at room temperature. After plating the cultures in each instance they were incubated at  $37^{\circ}$  C. for 24 hours. The plates made from the mixture of organisms and saliva after  $\frac{1}{2}$  hour showed 70 small green colonies; those made at the end of  $3\frac{1}{2}$  hours showed 20 colonies and in those plates made at the expiration of  $17\frac{1}{2}$  hours no colonies were present. In other words in a single experiment no pneumococci developed after being in contact with normal saliva for  $17\frac{1}{2}$  hours. The result suggests the presence in saliva of a pneumococcidal substance. The experiments I have been able to make are, however, too few to justify final conclusions.

<sup>1</sup> *Jour. Infect. Dis.*, 1907, 4, p. 629.

## SUMMARY.

In lobar pneumonia pneumococci isolated from the blood stream are insusceptible to the action of normal opsonin, while the great majority of those obtained from pneumonic sputum are readily phagocytatable under the influence of normal serum.

In 127 strains of pneumococci isolated from sputum of 26 cases of lobar pneumonia 93.7 per cent were phagocytatable hence non-virulent, and 6.3 per cent were non-phagocytatable hence virulent. The duration of the disease seemed to make no appreciable difference in the virulence of the organisms isolated. Cultures from the tonsils, the trachea, and the bronchi, even in fatal cases, showed no increase in the percentage of virulent strains as compared with cultures obtained from sputum.

This fundamental difference between the pneumococci in the blood and in the sputum and respiratory tract in pneumonia merits further investigation.

## THE TREATMENT OF GONOCOCCUS ARTHRITIS BY INJECTIONS OF DEAD GONOCOCCI, AND THE CLINICAL REACTION WHICH FOLLOWS THE INJECTIONS.\*

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It is now quite generally recognized that the course of certain infectious diseases may be favorably influenced by the subcutaneous injection at suitable intervals of small quantities either of the dead culture of the infecting organism, or of its products. This method of therapy is still in the early experimental stage, especially in diseases other than tuberculosis, and many observations will be required before we can form any definite conclusions as to its value. Infections by the colon bacillus, the staphylococcus, and the streptococcus have been treated by the subcutaneous injection of dead cultures often with apparent benefit, but by far the most extensive work along this line has been done with tuberculin in tuberculosis, in which the results have been on the whole encouraging.

The effect of the treatment of gonococcus infections by the injection of suspensions of dead gonococci has been recorded by a number of observers. Gonococcus vulvo-vaginitis has been treated by this method with varying success. Some workers have seen somewhat favorable results in the shortening of the duration of the discharge, while others have been able to detect no difference in the course of parallel series of cases, with and without the use of inoculations. A number of cases of acute urethritis in adults have been treated by killed gonococci, but in general the results obtained so far have not demonstrated any marked clinical value in this class of cases.

Relatively few cases of gonococcus arthritis and periostitis treated by inoculation have so far been recorded. Cole<sup>2</sup> and Meakins reported 15 cases of gonococcus arthritis in which inoculations were given and systematic observations made upon the opsonic index. After a careful study of the cases they concluded that inoculation of dead

\* Received for publication April 14, 1908.

<sup>2</sup> *Johns Hopkins Hosp. Bull.*, 1907, 18, p. 223.

gonococci appeared to have a decided value in favorably influencing the course of the disease.

The lesions which occur in the course of generalized gonococcus infections present certain characteristics which render these cases peculiarly favorable for the observation of the clinical effects of therapeutic injections of dead cultures of gonococci. The course, even in the acute cases, extends over a number of days at least, and much more often over weeks and months. The fever and other constitutional disturbances are relatively less than in other infections of corresponding extent in the body, and the lesions, involving chiefly the joints, tendons, and periosteum, are readily accessible to examination and careful observation.

In the course of the observations upon which this paper is based, 40 cases of gonococcus infection have been studied, including 31 cases of arthritis. The diagnosis of each case included in the series was determined beyond any reasonable doubt, from the clinical history and course as well as from the associated genital infection. In all the cases in any way obscure the diagnosis was confirmed by the isolation of the gonococcus from the joints, and in two cases from the blood.

In this connection it may be noted that there are many cases of obscure joint, periosteal, and synovial disease to which the term "chronic rheumatism" is applied, in which the gonococcus is the etiological factor. The remoteness in point of time of the primary infection, the peculiar clinical manifestations of the disease often simulating other affections such as tuberculosis, arthritis deformans, or other chronic arthritic or muscular diseases, the occasional extreme latency of the infection which becomes localized in some point of slight recent trauma with no detectable constitutional disturbance, and the tendency of physician as well as patient to regard an apparently healed gonococcus urethritis as a closed incident, all combine to cloud the diagnosis in these cases.

#### CULTURES USED FOR INOCULATION.

Bacteriologists are by no means agreed as to the relationship of the gonococcus to the meningococcus and other gram-negative diplococci, nor indeed as to the extent of the variations which occur in the various strains of the gonococcus. In working with a number of

cultures of the gonococcus, certain slight variations in cultural characteristics are observable, even in cultures of approximately the same age, and in the older cultures a gradual adaptation to growth on less highly albuminous media is frequently seen. Teague and Torrey in a recent study of the inter-relations of seven strains of gonococcus by means of the "fixation of complement" find marked differences in the immune sera obtained by the injection of the several strains into rabbits.

These and other similar facts have an evident bearing on the treatment of gonococcus infections by inoculation. In order that the inoculation treatment may have a general practical clinical value in the event of the experimental results receiving further confirmation, the dead cultures must be prepared, at least for the present, from heterologous organisms; for in many cases it is not only impracticable but impossible to obtain the so-called homologous strain.

Accordingly, for purposes of uniformity, all the cases in this series were treated with cultures obtained from other sources than the patients in question. The cultures of the gonococcus used were isolated from various lesions including urethritis and arthritis. As is well known, difficulties arise in the cultivation of the gonococcus which do not occur in the case of streptococci and staphylococci. Freshly isolated cultures of the gonococcus are often cultivated with difficulty; older cultures, however, usually grow readily, particularly on the more highly albuminous media such as ascites- and blood-agar.

If care is taken to transfer the cultures before the surface of the medium becomes dry, the organisms can usually be kept alive. Thus one strain has been preserved for 18 months and another for 12 months. However, in the preparation of material for inoculation, the more recently isolated strains have been used, preferably when from two weeks to three months old.

To obviate, in part at least, certain objections to the use of heterologous organisms, some of the material was prepared from two and sometimes three strains of the gonococcus. In many cases, however, but one strain was used. In a clinical study of this nature it is a matter of extreme difficulty adequately to compare the results obtained with different kinds of killed gonococci. The general impression is

that the immunizing responses were as good when one strain was used as when two or three were combined.

In preparing the killed gonococci the surfaces of large tubes of ascites- or blood-agar were inoculated, incubated for 24 to 48 hours, the growths then washed off with small quantities of salt solution, and the resulting suspension heated for one hour at 60° C. The number of organisms in the suspension was estimated by Wright's method, and also by employing the ordinary apparatus used in making a blood count. In the latter method the same line of procedure is followed as in making a red-blood count. Toisson's solution containing somewhat more methyl violet than usual is carefully filtered and used as the diluent. If a thin cover-glass is applied to the counting chamber, the stained organisms are readily distinguished with No. 7 Leitz objective. With care in agitating the pipette, a uniform distribution of the organisms can be obtained.

Lysol in salt solution was added to the suspension in sufficient quantity so that the final product consisted of a suitable number of organisms suspended in a solution of  $\frac{1}{4}$  per cent lysol. The dilution of the suspension was so gauged that the volume of the dose to be injected was from  $\frac{1}{2}$  to 1 c.c. Finally sterility was determined by cultures.

In the early cases treated, from 20 to 50 million organisms were given at a dose. A dose of this size usually produced a rise in the opsonic index, but often no coincident change occurred in the clinical course of the disease. It seemed advisable to increase the dose, and accordingly in the later work, from 100 to 500 million organisms were given. The intervals between injections varied from three to seven days.

#### THE OPSONIC INDEX.

The gonococco-opsonic index in 15 cases of gonococcus infection was studied with a view to determining its range and the fluctuations which may occur spontaneously and in response to treatment. In arthritic cases, the index before treatment was usually less than 1.0. A case of extensive arthritis gave indices of 0.6 and 0.9, a second case 0.5 and 1.1, a third 1.1, 0.8, and 0.8. In two other cases of somewhat less severe arthritis preliminary indices of 1.6 and 1.3, and of 1.3 were obtained. The average index of the arthritic cases

examined before treatment was 0.8. The more severe cases gave the lower average indices. In three cases of urethritis, without demonstrable metastatic lesions, the indices were 1.1, 1.1, and 1.2; 1.4 and 1.7; 1.5, 1.3, and 1.4 respectively. The injection of dead gonococci was followed usually by a sharp rise, the index sometimes reaching 2.5 or 3.0, with an average of from 1.5 to 2.0. Occasionally the rise was preceded by a slight fall. Similar observations have been made by other observers, and the results are cited here in further confirmation only of the general proposition that in gonococcus infection, as in other infections, the opsonic index shows certain characteristic variations, being often depressed during the course of the infection, and usually raised by the injection of suitable doses of dead cultures of the organism concerned.

Aside from the injection of dead gonococci, there are of course other factors which may cause fluctuations in the opsonic index. A patient with gonococcal prostatitis and a chronic urethral discharge received massage of the prostate. His gonococco-opsonic index as determined on five preceding days was 0.8, 0.8, 1.2, 0.8, 0.9. On the next day the prostate was massaged, and the following day the index was found to be 2.3. A week later, after the index had returned to 1.2 the prostate was again massaged, and 18 hours later the index was 2.9. Another patient suffering from gonococcus arthritis received massage of one of the knees involved, with a resulting definite rise in the opsonic index. Like observations have been made by Wright and others in various other infections, the specific opsonic index rising after exercise of joints, massage, the application of bandages to infected parts, etc.

Spontaneous fluctuations in the index also occur. Thus the index in a case of gonococcal epididymitis was determined for a number of days before treatment was begun. It was found to range between 1.0 and 1.3, and then suddenly rose to 2.9, falling 2 days later to 1.4, but with no corresponding variation in clinical course. The index in a case of arthritis was 0.8 on two successive days, and two days later without assignable cause it rose to 1.8. It is of course recognized that the technic of the determination of the opsonic index involves a certain margin of error, varying to a considerable extent with the skill and experience of the worker, but this margin of error

can hardly account for some at least of the fluctuations occurring in an otherwise regular series of gonococco-opsonic determinations. It seems probable that the explanation may be some accidental trauma or motion of joints, etc., similar in effect to massage of prostate or joints which we know at times produces just as marked rises in the opsonic index as does the injection of dead organisms. Owing to the localization of the infection in the joints which are necessarily exposed to more or less trauma, there is more likelihood in gonococcus arthritis than in at least some of the other infections of the occurrence of fluctuations in the index which cannot be correlated with the results of treatment, and which at times would be confusing if the opsonic index alone were the guide to treatment. The opsonic curve is the expression of only one of the many changes which may take place in the body fluids in response to infections.

Taking these facts into consideration, and bearing in mind that for some time at least inoculation therapy will depend for its control largely on clinical observation, it seemed advisable in the present work to use the clinical manifestations as the guide to the size and interval of the injections.

#### THE GONOCOCCUS REACTION.

In the following the clinical course of the cases under inoculation treatment, daily records of both subjective and objective conditions were made in so far as this was possible. It soon became evident that the course of events following the inoculation was not always one of progressive improvement. When seen 24 hours after the injection, patients repeatedly complained that the joint pains were worse rather than better. At this period of the work, the dose used was relatively small, and the exacerbation of the symptoms did not occur regularly after each injection, so that for a time, the phenomenon was regarded as an accidental coincidence. When the initial dose was increased, however, it was found that frequently the patients suffered from increased articular pain and tenderness after the first, often after the second, and occasionally after subsequent injections. Four cases in the same ward were given their first injections on the same day, the dose being 500 million. On the following day two of the cases showed only a slight increase in symptoms with some rise in temperature, and



pain in the joints. In these two, the arthritis was not extensive. In the other two cases in which the joint involvement was greater, there was a decided exacerbation of symptoms. The involved joints became more acutely tender, and the fever which had previously not exceeded  $99^{\circ} 5$  F. rose to  $101^{\circ} 5$ , associated with general malaise. On the second day after the injections, the acute symptoms had subsided and the patients felt about as well as before the injections were given. During the succeeding days improvement was more rapid than before the injections, so that the net gain of the first week of treatment was greater than that of the week preceding. In these cases the clinical picture after injection was most striking, and was highly suggestive of the phenomena observed in the tuberculin reaction.

The clinical records of the cases treated before this observation was made were now examined, and in those in which daily records had been made it appeared that the characteristic reaction had occurred though by no means to so great a degree. In subsequent cases, it usually has been possible so to graduate the dose of gonococci as to produce the reaction at will.

A typical gonococcus reaction is characterized by a rise in temperature, often only slight, an increase in pain and tenderness in the affected joints, with occasionally some increase in swelling, and a variable degree of malaise. The symptoms follow the injection in from 8 to 12 hours, and commonly last about 24 hours. Frequently there is a decided tenderness at the site of the injection, greater than occurs after the inoculation of the same dose of the same preparation in normal subjects. Occasionally there is marked redness and edema lasting from 24 to 48 hours. In a case of periurethral abscess of gonococcal origin without secondary infection which was under surgical treatment with drainage, an injection of 500 million cocci was followed in 18 hours by a moderate swelling and tenderness at the site of needle puncture, and also a marked increase in redness and tenderness about the wound. There was no coincident retention of pus, or local secondary pus infection to account for the phenomenon, and the wound returned to its normal condition in 24 hours. There is usually a slight increase in leucocytosis in the first 24 hours after injection.

The degree of the reaction is influenced by several factors. After

small doses (20-50 million in the cases in question) the reaction was not marked, and in a number of the early cases treated, it either did not occur or at least was overlooked. In one case (case 1), however, in which a 50 million dose was given, there was a reaction after each of the first three injections. When, beginning with a small dose, gradually increasing doses were employed, the reaction did not appear so typically, or was scarcely observable. When in a given case the same moderately large dose (300-500 million) was used for all the injections, the reactions became less with each injection. In four instances the reaction followed the initial dose only.

The most constant feature of the reaction is the increase in joint pain and tenderness. The temperature rise is often so slight as of itself to excite but little remark, especially in those cases in which there is a daily temperature of  $99.5-100^{\circ}$ . In opsonic terminology, the clinical reaction corresponds to the negative phase.

#### THE AMOUNT INOCULATED.

In the absence of definite knowledge as to the relative value of various strains of the gonococcus for producing immunizing responses, it is not possible to set any arbitrary number of killed gonococci as the proper dose to employ. Cole and Meakins used from 300 to 1,200 million. The dose employed by several workers in the treatment of vaginitis in children is much smaller (10-50 million). For adults a small dose might be placed at 50 million, a medium dose at 300-500 million, and a large dose at 1,000 million. In determining the dose for any particular case, the general condition of the patient, and the extent of the arthritis must be taken into account. The dose of 500 million in the two extensive cases cited above was apparently too large, whereas in the less extensive cases it proved to be quite harmless.

The best results as to treatment in this series were obtained when the initial dose was just large enough to produce a slight clinical reaction, and the subsequent doses increased gradually. In no case was there any evidence that harm followed a moderate reaction.

#### CLINICAL RESULTS.

The clinical course of gonococcus infections is extremely variable. The large majority of the arthritic cases recover spontaneously, though frequently convalescence is long and tedious, and the patient may be

partially disabled for months. A periostitis of the os calcis, or a chronic periarthritis of the wrist, though relatively slight, may totally incapacitate the patient for work. It is obviously fallacious to draw conclusions as to the value of a treatment from the observation of isolated cases, and even when a number of cases of a disease so variable in its course as gonococcus arthritis are grouped together, one must be very guarded in the conclusions as to the rôle of the treatment in effecting a cure.

In summarizing the results, the previous duration of the arthritis, its course in the days or weeks immediately preceding treatment, and the rapidity of subsequent improvement are of greater weight than the mere statement of the time required to effect a cure.

In general, inoculation gave better visible results in the chronic than in the acute cases. In several of the acute cases improvement was apparently more rapid immediately following the injections than before. But, while the acute cases terminated favorably and without undue delay, it must be recognized that they might have done so without treatment, though possibly less rapidly.

The evidence in favor of the inoculation treatment is stronger in the subacute and chronic cases. In a number of cases in which after a typical acute course there remained more or less indolent swelling and tenderness in one or more joints, after the first injection the signs of the infection subsided rapidly. A patient (case 26) had had a typical gonococcus arthritis for 10 weeks. For six weeks before inoculation there had been no improvement in spite of rest and medicines. He was able to hobble around, but the pain in the lower dorsal region, sternoclavicular joint, and in the heels rendered him unable to work. After the slight reaction following the first injection of 300 million cocci his pain was much less, and after two more injections at intervals of four days he was discharged with no pain or other disability. When seen two months later he was still free from the trouble. A number of similar cases might be cited.

The treatment of several very chronic cases was undertaken. A patient (case 10) had suffered for years from recurrent attacks of "rheumatism" in the region of the lumbar spine and in the heels. For seven months, with the exception of one month, he had been unable to work. At one time the heels, at another the back seemed

to be the worse. He had taken medicines of many kinds, electrotherapy, etc., without benefit. The prostate was large, tender, and the fluid obtained by massage contained typical gonococci. Inoculation of dead gonococci was followed by an increase in pain and tenderness in the affected parts for 24 hours and then by improvement. After six injections during a period of one month, his condition was very much improved and he was able to go to work. Each injection was followed by an exacerbation of local tenderness. The subsequent improvement was much slower, but progressive. The injections were continued with increased dosage, combined later with massage of the prostate which was much less tender than at the outset of treatment. At the end of three months' treatment recovery was practically complete. The later injections were not followed by any observable clinical reaction.

In four of the 31 cases in the arthritic series, the results were unsatisfactory. Two of these (cases 18 and 22) were acute cases in which but two injections were given because of peculiar obstacles to treatment. These might reasonably be omitted from the series. Another subacute but extensive case (case 2) received only small doses (50 million) for one month without improvement, and the treatment was discontinued. Massage of the prostate was substituted, with subsequent recovery after a slow convalescence.

The fourth case was that of a chronic alcoholic, who had also a chronic nephritis with considerable edema. In addition to his gonococcus arthritis, he had an otitis media, and a dacryocystitis of a year's duration. Occasionally a slight reaction could be obtained after inoculation, with temporary improvement, but he suffered repeated relapses, with the involvement of new joints, and the treatment was finally discontinued.

The general impression derived from a study of the series of cases as a whole is that inoculation was of material value in hastening recovery. The evidence is strongest in the more chronic ambulatory cases in which the benefit of rest in bed can be eliminated.

Certain other general therapeutic measures also contributed to the favorable results. The repeated withdrawal of joint fluid in large recurring effusions was no doubt of value. Only the smallest degree of fixation of joints was used. There were no cases in which

ankylosis occurred. Prostatic massage has long been recognized as a valuable measure in gonococcus arthritis. It is highly probable on clinical grounds as well as from its effect in raising the opsonic index that prostatic massage results in an autoinoculation similar in its effect to the injection of killed gonococci. In several cases in which, for purposes of observation of the unaided effects of inoculation, systematic massage of the prostate was omitted, the condition of the prostate improved coincidentally with the improvement in the arthritis after inoculations.

#### THE CLINICAL GONOCOCCUS REACTION IN DIAGNOSIS.

As already stated the clinical phenomena which follow the injection of dead gonococci in patients suffering from gonococcus arthritis are in many respects similar to those which follow injection of tuberculin in cases of tuberculosis. The rise in temperature and malaise are relatively less in the gonococcus infections, but there occurs in addition an increase in symptoms in the joints. In some instances there is, too, a local reaction at the site of injection with tenderness, occasionally redness, and some edema. These local signs commonly subside in 24 hours, though in three cases they persisted for three or four days. Great care has been taken throughout to insure surgical cleanliness in the giving of the injections. Moreover, in a number of normal individuals, to whom the same amount was given, no local reaction was noted.

The frequency with which these clinical phenomena occurred suggested the possibility of utilizing the reaction in the diagnosis of obscure cases of arthritis in which the gonococcus was the suspected cause. The effects of the injection of dead gonococci into patients not suffering from gonococcus infection were accordingly studied. Eight adults in whom there was no history or sign of gonococcus infection were given injections of 500 million dead gonococci. In none of these was there any local change other than that following an ordinary hypodermic puncture, and no fever or constitutional disturbance was observed. In a case of pyorrhea alveolaris with subsequent general infection, and painful swellings over the extremities, there was no increase of fever or local symptoms following the injection. A case of gout with active joint involvement showed no local or general change after a dose of 500 million. Leucocytes before

injection 13,200; 18 hours after injection 13,000. Temperature normal throughout. A case of articular rheumatism showed no reaction, after 500 million. There was no increase in leucocytosis and the temperature chart showed no abnormal variations. A case of acute arthritis with pericarditis was thought possibly gonococcal in origin. There was no reaction after a dose of 500 million. Cultures from the blood and from a small amount of clear fluid aspirated from the knee remained sterile and the prostatic fluid contained no gonococci. The subsequent course was typical of acute rheumatic fever. In four other cases of acute and subacute articular rheumatism there was no reaction after injections of 500 million cocci.

In a number of suspected gonococcus cases the reaction was of value in making an early diagnosis. A case of monoarticular arthritis with effusion in the knee in which gonorrheal infection was denied was given an injection of 500 million. The evening temperature, which had previously reached only 100, rose to 101°·8 and the joint pains increased. The knee was aspirated and the gonococcus isolated in pure culture from the fluid. A case of chronic arthritis which had resisted all treatment was given an injection. A slight rise in temperature with some increase in joint pains followed. The prostatic fluid was found to contain gonococci, and the subsequent course was that of gonococcus arthritis. A patient who had suffered from extensive gonococcus arthritis had been bed-ridden for one year. There was practically no motion in the knees. After an injection of 500 million the temperature which for weeks had been normal rose to 99°·5, and the patient complained of malaise and increased pain in the joints. A patient with aortic aneurysm, who denied gonorrheal infection, had been selected for control experimental inoculations. After an injection of 500 million cocci, the temperature which had been uniformly normal, rose to 100° F. without any other apparent cause, returning to normal next day, with no subsequent rise. The prostate was examined and found to be large, somewhat tender, and the secretion contained numbers of leucocytes with typical intracellular gonococci.

#### SUMMARY.

Systemic infections by the gonococcus tend to spontaneous recovery. In many of the cases of gonococcus arthritis, the clinical

manifestations are acute, and the course extends over a few days or weeks. Certain cases, however, do not recover so quickly, and the condition passes into a chronic stage which may last months or years. In the first class of cases immunity develops rapidly, and clinically little benefit appears to result from the injections of dead gonococci. In the chronic cases, the mechanism of immunity fails to rid the body of the organisms, and they persist either in the original lesions or in one or more foci such as the prostate and give rise from time to time to new lesions at various points of localization. When dead gonococci are injected a reaction frequently follows with an increase in constitutional and local symptoms. This reaction is later followed by a period of improvement. The fact that with a dose of constant size, the reactions become less with each injection would seem to be a strong argument in favor of the value of the injections in hastening the development of immunity. Clinically in a number of cases the injections of dead gonococci have seemed to be of distinct value.

Many more series of cases must be studied before a definite opinion can be expressed, but the results obtained thus far seem to indicate that in certain cases at least of gonococcus arthritis recovery can be hastened by the injection of dead gonococci. No harm has appeared to follow the injections, and it is possible that the use of larger doses will be found desirable in some cases.

With further work the limitations as well as the advantages of the method will appear, and it should be recognized, that, while it is attractive theoretically as a specific therapeutic measure, too much must not be expected of it in the way of marvelous cures. It should be used rather in conjunction with other general measures such as rest, aspiration of joints distended with fluid, massage of the prostate, and other surgical and general hygienic treatment.

The reliability of the clinical gonococcus reaction as a diagnostic procedure will also be determined only after many tests. It has many points in common with the tuberculin reaction, and similarly too, there may well be cases of gonococcus infection found which do not respond. It appears, however, to be well worth a trial. Should the reaction prove to be reliable, a valuable and much-needed aid will be at hand for the diagnosis of obscure joint, synovial, and periosteal diseases.

Until recently it has not been deemed expedient to carry on in experiments on certain other phenomena of hypersusceptibility gonococcus infections such as the ophthalmic reaction, though this work has now been begun. It has already been noted that the local reaction after the injection of dead gonococci is greater in cases of gonococcus infection than in normal individuals.

I am greatly indebted to Dr. Hektoen of the Memorial Institute for advice and guidance during the preparation of this paper, and to the attending and house staffs of the Presbyterian and Cook County Hospitals for the opportunity afforded to observe clinical cases and for assistance in carrying out the treatment. I wish also to acknowledge the valuable assistance of Drs. A. H. Curtis and R. C. Menzies of the County Hospital who devoted much time and care to the daily observation and recording of the clinical phenomena of the cases in their charge.

Abstracts of the clinical histories of several of the cases are appended for the purpose of illustrating the phenomena observed during the course of the treatment.

The principal facts in the arthritis cases are summarized in the table. In a number of the cases, particularly the earlier ones, the records were not definite as to the occurrence of a reaction after the injections, and in these instances the fact is indicated by a dash (—) under the heading "Clinical Reaction."

The duration of treatment as given in the table comprehends the time elapsing until recovery was clinically complete, or until the patient passed from observation, in which event note of the fact is made.

#### ABSTRACTS OF CASES.

CASE 1.—M. S., female, white, *aet.* 27, actress; entered the hospital March 30, 1907, complaining of pain in the left knee, both ankles, left hip, elbows, right wrist, and right thumb.

The following history was obtained: Fifteen months ago the patient had severe pain and swelling in left knee joint lasting one month, associated with slight fever. Two months later a pelvic abscess formed and was drained.

Four weeks ago the patient fell, striking upon the left knee, but, aside from slight tenderness, suffered no inconvenience until one week ago when the knee became swollen and acutely painful. This was followed by pain and some swelling in both ankles, left hip, elbows, right wrist, and several of the small joints of the right hand. There had been only slight fever, no sweating, and no other marked constitutional



disturbance. Previous to marriage the patient was in good health. No history of rheumatism or other infectious disease. Following her marriage 8 years ago, the patient had 2 miscarriages, at 4 and 5 months, 1 full-term child, who has always been delicate, 1 miscarriage, 1 full-term healthy child, 1 miscarriage. No history of rash or other specific lesions. No further history of blennorrhagic infection could be obtained from the patient.

Physical examination showed a fairly well-nourished young woman. Nothing of note was found in the head, neck, or chest. The heart outlines and sounds were normal. The upper abdomen was normal. A vaginal examination showed dense bilateral pelvic adhesions, but no pus focus or tenderness was made out. There was a slight leucorrhea but careful search revealed no gonococci in the discharge.

The left knee was held in partial flexion, and there was marked tenderness and some swelling and redness in the popliteal space and over the inner flexor tendons. No effusion in the joint. The right wrist was slightly swollen, reddened, and exquisitely tender. There was pain on motion in the ankles, left hip, and in the elbows. The interphalangeal joint of the right thumb was painful, slightly swollen, and red.

A blood examination showed 4,160,000 red cells, 12,000 leucocytes, and 67 per cent hemoglobin (Dare). The urine contained a few leucocytes, but otherwise nothing abnormal was noted on careful examination.

On admission the patient was given salicylates by the house physician, and the affected joints partially fixed by soft bandages. Four days later the salicylates were stopped and the patient given potassium iodide gr. x and mercury biniodide gr.  $\frac{1}{10}$  three times a day. Credé ointment was applied daily to the affected joints. Hot dressings were applied to the knee with some benefit in allaying the pain which was particularly severe at night. The evening temperature did not rise above 100° F. with a morning range of from 98° to 99°. There was no sweating and the constitutional disturbance was relatively slight compared with the local trouble in the joints.

Clinically the case appeared to be one of gonococcus arthritis. After 10 days' rest in bed under the above treatment there was very little improvement in the knee and wrist, the two joints most involved.

Gonococco-opsonic index April 5, 1.1; April 6, 0.8.

*April 10.*—Extreme tenderness with swelling over median aspect of knee joint, and in the popliteal space. Some swelling and pain on motion in right wrist. Pain on motion, slight redness and swelling of left shoulder joint. Maximum temperature for past two days 99°8 F.

Gonococco-opsonic index, 0.8. A subcutaneous injection of 50 million dead gonococci (Preparation I) was made into the left arm.

*April 11.*—Some increase in tenderness and swelling in right wrist. Maximum temperature 100°8 eight hours after injection. Opsonic index 1.2.

*April 12.*—Still pain and swelling in right wrist. Knee and shoulder somewhat better. Max. temp. 99°6.

*April 13.*—Opsonic index 1.0. Leucocytes 18,500.

*April 15.*—Wrist still swollen and tender. Knee somewhat better. Opsonic index 1.1. Injected 50 million cocci (Prep. I). Max. temp. eight hours after injection 101°2.

*April 16.*—Wrist more swollen and tender. Max. temp. 100°8.

*April 17.*—Opsonic index 1.9. Wrist improving; no spontaneous pain. Knee and shoulder can be moved without pain. No swelling. Max. temp. 99°8.

*April 19.*—Wrist again painful, with some increase in swelling. Max. temp. 99°c.

*April 20.*—Wrist about as yesterday. Some pain in knee. Opsonic index 1.5. Injected 50 million cocci (Prep. I). Max. temp. 99°2.

*April 22.*—Opsonic index 2.2. Considerable improvement in wrist. Can move fingers better than previously.

*April 24.*—Wrist better. Can move fingers easily. Knee joint can be moved without pain. General condition good. Max. temp. 98°8. Leucocytes 13,500. Opsonic index 1.2. Injected 50 million cocci (Prep. I). Max. temp. eight hours after injection 98°6.

*April 25.*—Practically no change in joints.

*April 27.*—Some stiffness in left knee, but no swelling nor tenderness. Is using right hand and wrist normally without pain.

*May 1.*—No trouble in any of joints. Injected 50 million cocci (Prep. I). Max. temp. eight hours after injection 99°0.

*May 4.*—Insisted on returning to work. Walked out of hospital in good condition without joint symptoms.

In this case the diagnosis of gonococcus arthritis was apparently confirmed by the clinical course, although the gonococcus was not isolated from any of the lesions. The infection was one of moderate extent and severity, and for the first ten days the joints did not materially improve with rest in bed and medicinal treatment. The strain of gonococcus used was isolated five months previously from a case of acute urethritis, and kept in culture by repeated transfers on ascites-agar. Following each injection there occurred a rise in the opsonic index, though the opsonic index determinations were not so frequent as could have been desired. After each of the earlier injections there was a distinct rise in temperature, followed a few hours later by an exacerbation in the joint symptoms. The clinical import of these phenomena was not fully recognized at the time, but later, on referring to the clinical record, they were seen to occur with considerable regularity. It is difficult to say in how great a degree recovery was accelerated by the use of the inoculations. It may be stated, however, that the course was shorter than one would expect in cases of like extent and severity.

CASE 3.—J. B., male, *aet.* 29, laborer.

*December 19, 1907.*—The patient applied for dispensary treatment, complaining of pain in the mid-dorsal region, tenderness and pain on motion in the left shoulder and right knee.

*August 13, 1907.*—Following exposure a urethral discharge appeared with dysuria followed in one week by pain in back, left shoulder, and right knee in rapid succession. There was fluid in the knee joint for three weeks. The patient was confined to bed for four weeks. The highest temperature was 100° F. For the past three months has been able to be up and about, but unable to work on account of stiffness and pain in the joints.

Two years previously had an operation for frontal sinus infection. Otherwise the previous history was unimportant.

On examination there was found marked tenderness in the left shoulder without swelling, and over the mid-dorsal vertebrae, with pain on flexion (present since onset of trouble). Tenderness and pain on motion in right knee, but no effusion. Heels tender on pressure. Tenderness on pressure over an area 1 cm. in diameter over the crest of left ilium, apparently in the periosteum. No urethral discharge. The prostate

was large, tender, and after massage a considerable amount of fluid was expressed, which contained leucocytes and intracellular gram-negative biscuit-shaped diplococci. Temperature 99°. Pulse 90. The urine obtained before massage of prostate was normal.

A diagnosis of gonococcus arthritis and periostitis was made and the patient was given a subcutaneous injection of 100 million cocci (Prep. V) on December 19. He was also given urotropin gr. v four times a day.

*December 23.*—Patient reported that on the day following the injection the pain in all the affected joints was worse but that on the second day there was considerable improvement. On examination the joints were found to be less painful than at the previous examination; no tenderness in the heels; the tender area over the left ilium had disappeared. The patient stated that there was a slight morning discharge. A simple tonic was substituted for the urotropin, and a subcutaneous injection of 300 million cocci (Prep. VI) was given.

*December 30.*—Patient reported that he was again worse for two days after treatment, but now felt so much better that he desired to return to work. Some tenderness in dorsal spine, and in left heel. Injected 300 million cocci (Prep. VIII).

*January 6, 1908.*—Reported only slight exacerbation of symptoms after injection. Occasional pain on pressure in left heel. Some stiffness in left shoulder but no pain on passive motion. Back still somewhat painful on forward flexion. Injected 300 million (Prep. X).

*January 16.*—No increase in symptoms after last injection. There was now no complaint of pain in any of the joints or heels. There was some soreness in the lumbar muscles but no tenderness could be elicited in the vertebrae on pressure or motion. Injected 300 million (Prep. X). The patient returned to work.

This patient had taken various medicines for rheumatism without benefit, and had been incapacitated for work for a period of four months. The duration of injection treatment was about four weeks. No attempt was made to place the affected joints at rest.

CASE 6.—H. H., male, *aet.* 38, cook. Illness began October 15, 1907, with pain and swelling of the left ankle. Two days later the left knee became swollen and extremely painful on movement. The pain and swelling in the ankle quickly subsided but the knee did not improve and the patient entered a hospital.

Previous to the onset of the arthritis the patient had a sore throat for two days. No chills and not much fever. There was a history of syphilis 14 years previously, with a subsequent alopecia. Gonorrhea four years previously with a discharge for four months but no apparent complications. Since then there had been no discharge. No other illness.

The left knee was greatly swollen and tender with but little redness of the skin. The swelling was due partly to fluid in the joint, but there was also considerable swelling of the periarticular structures. The other joints, including the left ankle, appeared normal. The prostate showed little if any enlargement, and was not tender. The prostatic fluid showed no abnormalities microscopically. The remaining physical examination revealed nothing of note.

Urine normal; blood: reds 4,100,000, hemoglobin 78 per cent (Dare); leucocytes 9,000.

The patient remained under hospital treatment for one month, with rest in bed, partial fixation of the knee by bandages, salicylates, iodides, and mercury. The

temperature ranged from normal in the morning to 99° F.-100° F. in the evening. During the last week of this period the temperature did not rise above 99° F., but there was no improvement in the condition of the knee.

*November 14.*—The prostatic secretion obtained by massage showed a few organisms which resembled gonococci.

*November 16.*—The knee joint was aspirated and 60 c.c. of cloudy yellow fluid obtained, which contained many polynuclear leucocytes, and a few diplococci. The gonococcus was obtained in pure culture from the fluid.

*November 20.*—One hundred million dead gonococci (heterologous) were injected subcutaneously. A slight rise of temperature followed; but no local reaction in the joint. Leucocytes just before injection 10,500; 18 hours after injection 13,500.

*November 25.*—Two hundred and fifty million cocci were injected. No reaction whatever was observed. There was very little change in the condition of the knee. Leucocytes just before injection 8,400. Leucocytes 18 hours after injection 9,500.

*December 3.*—Sixty c.c. of slightly cloudy fluid was aspirated from the joint. A few gonococci and many polynuclear cells were present in the fluid; 500 million cocci injected subcutaneously. No reaction was observed.

*December 10.*—The periarticular swelling had decreased, but fluid had reaccumulated; 60 c.c. of slightly blood-stained gelatinous fluid aspirated. This fluid was clearer than that from preceding aspiration. Five hundred million cocci injected (Prep. VI). Following this injection the evening temperature which had been 99° F. on two preceding days, reached 99° 8 F. on the two succeeding days. Seven days later the evening temperature was normal, the swelling of the knee was decreased, and the patient was able to walk without pain, although the motion was not as free as normal. The patient left the hospital and returned for inspection at intervals of seven days. He was given two more injections of 500 million (Prep. VI), Dec. 17 and Dec. 24.

*January 10.*—There was no swelling or pain in the knee, and motion was normal without stiffness. When last seen, January 20, there had been no recurrence of trouble.

The favorable result in this case was due in part, no doubt, to the repeated removal of the fluid from the joint, although the infection involved to an unusual extent also the periarticular structures. Throughout the treatment the joint was allowed free movement, no apparatus for fixation being used. Duration of treatment, 34 days.

The limitation of the infection to one joint probably accounted for the relatively slight reaction following the injections. A slight rise in the leucocytes, followed each injection.

CASE 7.—J. T., male, *aet.* 36, laborer. Patient came to the dispensary January 3, 1908, complaining of pain in left ankle and swelling and pain in the left knee. The pain was continuous and worse at night.

First had gonorrhea 14 years ago, followed three months later by "rheumatism" which involved the knees, hips, and ankles, and which lasted intermittently for two years.

*November 15, 1907.*—The patient had a recurrence of gonorrhea, and on December 1, began to have pain in the left ankle, followed in three days by swelling and pain in the left knee. Very little fever or constitutional disturbance.

On examination, the left knee was found greatly enlarged, due in part to periarticular swelling, and in part to a moderate joint effusion. Some swelling, redness, and tenderness were present over the left internal malleolus. No tenderness was elicited in the heels. A slight urethral discharge was present which contained typical intracellular gonococci. The prostate was normal in size and not abnormally tender; 120

c.c. of greenish-yellow cloudy fluid aspirated from the knee joint. Smears showed many polynuclear leucocytes, but no gonococci or other organisms were found. Cultures on ascites- and blood-agar remained sterile.

*January 3.*—A subcutaneous injection of 300 million cocci (Prep. VI) was given and the patient directed to take urotropin gr. v four times a day.

*January 4.*—Pain in the knee better (relieved by aspiration). Pain in ankle worse.

*January 6.*—Ankle some better but still tender. Knee less swollen and only moderately painful. 300 million cocci injected (Prep. X).

*January 9.*—Ankle more painful for 24 hours after last injection. Knee distended with fluid. Aspirated 120 c.c. of cloudy yellowish gelatinous fluid. 300 million cocci injected (Prep. X).

*January 13.*—Knee joint again distended with fluid. Aspirated 150 c.c. cloudy yellowish fluid from knee and a flannel bandage applied to joint. Ankle better. Very little swelling or tenderness. Urethral discharge decreased, but present; 300 million cocci injected (Prep. X).

*January 16.*—Knee much better; very little fluid, and not tender. Ankle which was worse for 24 hours after last injection slightly swollen and tender; 300 million cocci injected (Prep. X).

*January 20.*—No swelling or tenderness in knee. No increase in pain or swelling in ankle after last injection. Some tenderness and swelling still present in ankle. Some tenderness in left heel.

*January 23.*—Walked without cane. No fluid, tenderness, or stiffness in knee. Ankle completely recovered except for slight stiffness; urethral discharge had ceased; 500 million cocci injected (Prep. XI).

*January 30.*—No exacerbation of symptoms after last injection. A small amount of fluid present in knee; slight urethral discharge present; 500 million cocci injected (Prep. XI).

*February 6.*—No pain in joints. Knee and ankle freely movable; there was a slight increase in tenderness in ankle after last injection; 500 million cocci injected (Prep. XI). Also the patient was given 1 per cent protargol solution for urethral injection.

*February 13.*—The patient reported that he had returned to work and had no pain nor stiffness in any of his joints. Urethral discharge had ceased.

CASE 8.—J. M. C., male, *aet.* 36, cook. Gonorrhea first 15 years previously, with repeated subsequent attacks. Arthritis four years previously involving toes, ankles, and knees.

Present illness began two weeks previous to admission to the hospital with urethritis, followed one week later by arthritis involving successively, in order, the small joints of right hand, the right ankle, right knee, left shoulder, left great toe, left knee. When first seen, the patient had been under hospital treatment with rest in bed, iodides, and urethral injections for two weeks. The arthritis (of three weeks duration) had improved somewhat.

*November 13.*—The patient had become very thin during his illness. The left ankle was painful and swollen. Right wrist stiff and swollen. Tenderness and swelling in right knee and left great toe. Fluid in left knee; heels tender; prostate enlarged and tender. A smear from urethral discharge after prostatic massage showed typical intracellular gonococci. Injected 500 million cocci (Prep. VI).

*November 14.*—Pain in joints worse. Marked malaise. Slight rise in temperature.

*November 15.*—Joints still painful but some improvement.

*November 17.*—Joints showed marked improvement. Fluid in knee decreased.

*November 23.*—Improvement had been slow but continuous to this date. Knees and ankles still somewhat tender. No fluid. Tenderness but no swelling in right elbow and in shoulders. Some swelling over dorsum of both feet. Injected 100 million cocci (Prep. VI).

*November 26.*—Much better. Able to get up and move about ward. No reaction after last injection.

*November 27.*—Injected 300 million cocci (Prep. VI).

*November 30.*—Still some stiffness in right knee and right wrist. Injected 500 million cocci (Prep. VI).

*December 1.*—Involved joints sore and stiff. From this point on improvement was rapid. An injection of 300 million cocci (Prep. VI) was given on December 8. On December 10 there was no pain or tenderness in any of the joints, and the only complaint was of stiffness on attempted movement. No limitation of passive motion. The patient remained in the hospital 16 days longer and gained rapidly in weight and strength. On December 20 he reported for inspection. No limitation of motion or tenderness in any of the joints. Two months later the patient reported that he was in good health and that all the joints were normal.

In all, this patient received five injections. The duration of treatment was four weeks. The first injection of 500 million cocci was followed by a marked clinical reaction, with exacerbation of joint symptoms, malaise, and some fever. The arthritis was extensive and the patient showed the effects of prolonged illness by his emaciation. The dose in this instance proved to be too large as the degree of reaction was greater than was desirable. Subsequent smaller doses were not followed by marked reaction though the joints showed some increase in tenderness following a second later injection of 500 million cocci.

CASE 9.—A. C., male, *act.* 37, laborer. Entered the hospital complaining of pain and swelling in the left knee, which had been present for two weeks. Denied gonorrheal infection. A slight urethral discharge showed no organisms. The periarticular structures of the left knee were greatly swollen and tender. Fluid was present in the joint. Prostate normal in size and not tender. After two days' rest in bed there was no improvement. Following the injection of 100 million cocci (Prep. VI) the evening temperature which had previously been 100° F., rose to 101° 8'. Three days later a second injection of 300 million (Prep. VI) was given, and next day 25 c.c. semipurulent yellowish fluid was aspirated from the joint. The gonococcus was obtained in pure culture from the fluid.

Five more injections of 100, 150, 300, 300, and 500 million respectively were given at intervals of five days, each injection being followed next day by the aspiration of fluid from the joint. No rise in temperature followed the later injections.

The patient was discharged with good motion of the joint without pain or swelling. Duration of treatment 30 days. Incision and irrigation of the joint were considered, but the result justified the more conservative course.

In the light of previous cases, the rise in temperature following the first injection favored the probable diagnosis of gonococcus arthritis, which was later confirmed by cultures.

CASE 10.—E. G., male, *aet.* 36, machinist, single. When first seen in the office October 21, 1907, the patient complained of tender heels, pain and stiffness in the dorsal spine, and tender points over the right tibia just below the patella, the crest of the left ilium and in the right sacroiliac region.

The following history was obtained. With the exception of typhoid fever 18 years ago, the patient has suffered from no severe illness. No syphilis. Had an attack of gonorrhea 14 years ago, with a discharge for two weeks, followed by a recurrence soon after, the discharge persisting for five weeks. No arthritis.

Six years ago the patient suffered from pains in the dorsal region, particularly on bending over. This was worse in the morning and improved after moving about for an hour or so. The pain and stiffness in the back continued at intervals, with periods of remission until April, 1907, when he had a urethral discharge which disappeared after two days and has not been present since. He insists that there was no possibility of exposure to fresh infection. Immediately following the discharge the left heel and left hip became painful. Soon after, the right heel became tender. He was partially disabled for several weeks, and then returned to work. A month later the ankles and heels became painful, but there was very little swelling and no redness. The temperature and pulse were normal, and there was no other constitutional disturbance noted. A diagnosis of flat foot was made and the patient was given steel arches with but little resulting benefit. Three months ago he began to have pain in the left hip, extending down the back of the leg. This continued for two months and one month ago he entered a hospital where a diagnosis of sciatic neuritis was made. He received no benefit from two weeks' treatment.

During the course of his illness the patient had remained in good physical condition, but for seven months had been incapacitated for work, with the exception of a short time soon after the onset. He was an intelligent man, and while discouraged by his prolonged disability he was not neurotic, and did not magnify his symptoms. He had received many kinds of treatment, including electricity, thermotherapy, salicylates, iodides, and mercury. He was temperate in the use of alcohol and tobacco.

The physical examination showed a well-nourished young man, of medium height. Nothing abnormal was noted in the chest or abdomen. Both heels were very tender on pressure, but there was no swelling or redness. A tender area 1 cm. in diameter was present over the left tibia just below the insertion of the patellar tendon, with apparently a slight thickening of the periosteum. A small tender area was present in the right sacroiliac region which seemed to be in the fascia rather than in the bone or joint. Over the lower dorsal vertebrae there was tenderness on deep pressure on the spinous processes, but no lesion of the bodies of the vertebrae could be made out. There was no urethral discharge. The prostate was uniformly enlarged, rather firm and tender on deep pressure. An examination of the prostatic fluid obtained by massage showed a moderate number of leucocytes, a few typical gram-negative diplococci, both free and intracellular. Urine normal.

A diagnosis of gonococcus arthritis and periostitis was made and treatment by the injection of dead gonococci was begun. No other medication.

October 21.—Six hundred million cocci injected (Preparation VI) in right arm.

October 24.—The back was better, though still stiff. Tibia and heels as before. Soreness in the sacroiliac region had disappeared. The patient reported that for two days after the injection he felt worse. There was an increase in the pain and stiffness

in the back, and also a slight redness at the site of injection; 700 million cocci injected (Prep. VI).

*October 26.*—Not much change. There was very little increase in tenderness after last injection; 700 million cocci injected (Prep. VI).

*October 30.*—Slight increase in pain in tibia after last injection. This was somewhat improved. The back was still painful, but less so than before treatment; 750 million cocci injected (Prep. VI).

*November 4.*—Not much improvement since October 30; 500 million cocci injected (Prep. VI).

*November 11.*—Patient stated that he felt better than for seven months. The pain in the back though still present was much less and he could bend forward more easily. There was no increase in pain or tenderness after the last injection.

*November 15.*—Not so well. The left heel was tender, and there was pain in the back somewhat higher than before. The spine was only slightly tender on pressure, but there was some limitation of forward flexion on account of the pain. The prostate was somewhat tender and larger than normal. Prostate massaged.

*November 20.*—Back better. Still some pain in heel; 200 million cocci injected (Prep. VI).

*November 25.*—No reaction noted after last injection, 200 million cocci injected (Prep. VI).

*November 30.*—Two days ago the pain in the back was worse, but this disappeared in 24 hours and the patient reported that he felt better than at any time previously, and was able to return to work.

Up to this point the patient had improved steadily. The acute tenderness in the heels and back had decreased until the only complaint was that of slight pain in the morning on rising which disappeared in about two hours. The injections were continued at intervals of about five days, using an average dose of 300 million cocci for six weeks longer. At the end of this period there was only a very slight tenderness in the back. The prostate was still tender and the secretion contained a few gonococci. The injections were continued as before, and the prostate massaged. The improvement was very slow, but there was no relapse and by the middle of February the recovery was practically complete.

This case was of long standing and very stubborn. During the first month of treatment, the progress was satisfactory. It was a matter of much greater difficulty however, to get rid of the last traces of infection, particularly in the prostate, which seemed to be the focus of infection as is the case in so many of these patients. Massage of the prostate was purposely omitted during the early treatment, as it was desired to see to what degree the prostatic infection could be influenced by the injection of cocci subcutaneously. When prostatic massage was resumed, the prostate was smaller and much less tender than when treatment was begun, although there was some trouble remaining, as was evidenced by the presence of a few organisms in the expressed secretion. Throughout the course of treatment no constitutional disturbance was observed. Locally in the lesions, however, a distinct exacerbation of symptoms followed the injections. This reaction lasted 24 hours, and was followed by a period of improvement. The degree of the reactions became progressively less, though in this case an occasional slight increase in local tenderness occurred even after the later injections.

In point of the time required for treatment this case is not a brilliant example of the success of the therapy, though it seems not unreasonable to credit the method with



some part at least in the recovery. Throughout the treatment the patient led a normal life taking daily outdoor walks.

CASE 11.—O. H., male, *aet.* 25, laborer. Entered the hospital November 27, complaining of pain and swelling in the ankles and feet, of 10 days' duration, and of an acute urethritis, present for 5 weeks.

Five years previously had gonorrhea lasting five weeks, without complications. No other illnesses.

On examination the left foot was found swollen and tender, with some redness over the dorsum. Extensive involvement of the tarsal and metatarso-phalangeal joints. The right ankle, and right metatarso-phalangeal joint of the great toe were swollen and extremely tender. A purulent urethral discharge contained typical gonococci.

After three days' rest in bed the pain and swelling in the joints were no better. Two injections of 100 and 150 million cocci respectively at intervals of four days were followed by some improvement. After a third injection of 300 million four days later (December 8) there was a marked exacerbation of joint pain, lasting 24 hours, followed by a period of improvement. Two more injections of 500 and 300 million were given at intervals of seven days. The arthritis subsided slowly but steadily and the patient was able to walk without much difficulty by December 29. On leaving the hospital January 4 there was only a slight tenderness in the left foot.

The case was an acute one of moderate severity. Recovery, though occurring within a reasonable time, was not more rapid than seen in cases under other treatment, and could hardly be ascribed to the use of the injections. A decided reaction followed the third injection of 300 million cocci.

CASE 12.—M. F., male, *aet.* 38, engineer. Contracted gonorrhea 17 years ago, with subsequent attacks nine years, six years, and three years ago. Arthritis involving the ankles, right instep, both knees, and one finger followed the attack three years ago. The patient was sick for 14 weeks, and had to use a cane for a year afterward.

The present urethritis appeared October 2 and the arthritis October 9, involving both ankles and knees.

When first seen on November 13, the patient had been under hospital treatment with rest in bed for three weeks, without much improvement. Both ankles were swollen and tender; heels tender. Both knees painful on motion, with moderate effusion. Right sacroiliac joint tender. A typical purulent urethral discharge was present. Fever slight, normal in morning, occasionally reaching 100° F. at night.

November 13.—8 P.M.; 500 million dead gonococci were injected subcutaneously.

November 14.—Joints worse, acutely tender; general malaise with headache and anorexia. Temperature A.M., 100° F.; P.M., 101°.

November 15.—Better but still felt sick.

November 16.—Much better. Temperature P.M., 99°6. Joints much less tender.

November 17.—Considerable improvement in all joints except right knee which contained a large amount of fluid. No fluid in left knee.

November 20.—No demonstrable fluid in knees. Left knee, ankles, heels, and sacroiliac joints showed some tenderness.

November 23.—An injection of 100 million cocci was followed by a slight increase in pain in the affected joints, but there was no rise in temperature.

November 27.—An injection of 300 million cocci was given with no marked increase in symptoms.

*November 30.*—One hundred million cocci injected. Some increase in stiffness in joints but no constitutional disturbance.

The patient at this time was emaciated and weak. The arthritis though still present was much less painful. Improvement was steady but slow. Injections were given as follows: December 4, 150 million; December 9, 300 million; December 13, 250 million; December 21, 300 million; January 7, 500 million. No observable reaction followed these injections. On December 4 the patient sat up in a chair, and on December 16 was able to walk about the ward with the aid of crutches. His appetite improved and he gained rapidly in weight and strength.

When discharged on January 10, the patient had good motion in all the joints. With the exception of a small tender area over the inner aspect of the left knee, there was no pain or tenderness present.

This case was extensive, with evidences of severe toxemia. The emaciation and weakness were pronounced. For three weeks prior to the inoculations there had been little improvement with rest in bed. The first injection of 500 million was followed by a decided reaction, and it was evident that a smaller dose would have been better. Later, when convalescence was established, a dose of 500 million was followed by no reaction whatever. Here, as in other cases, it is impossible to state the time which would have been required for recovery on other treatment. The injection of dead gonococci did, however, have a decided influence on the clinical course of the disease, as evidenced by the reactions, and when suitable doses were employed, the effect appeared to be not unfavorable.

TABLE I.  
SUMMARY OF CASES.

Case No.	Age	Sex	Joints Involved	Original Infection	Pre-vious Arthritis	Date of Apparent Rec- infection before Present Arthritis	Duration of Present Arthritis before Treatment	Inocula- tions	Num- ber of Inoc- ulations	Clini- cal Reac- tions	Duration of Treat- ment	Result	General Treatment	Remarks
1	M. S. 27	F.	Knee, wrist, thumb	?	15 mo.	.....	2 wks.	50 mil.	5	3	24 days	Recovery	Rest in bed. Tonics	No improvement after 10 days' rest in bed prior to treatment. Rise in gonococ- cogenic index, coincident with periods of improvement after inoculations
2	McC. 27	M.	Knees, ankles, shoulder	5 yrs.	.....	3 mo.	10 wks.	75 mil.	6	3	1 mo.	No im- prove- ment	Rest in bed. Light flannel bandage to joints	Gonococcus obtained in culture from joint. No improvement with inoculations. Re- covered in 2 mos. with massage of prostate
3	J. B. 29	M.	Shoulder, dorsal vertebrae, knee, heels	4 mo.	.....	.....	4 mo.	250 mil.	4	3	4 wks.	Recovery	Placebo. Dispen- sary treat- ment. No fixation or rest of joints	Improvement rapid after injections. A chronic case, which had failed to im- prove after 4 mos. of other treatment
4	N. M. F. 29	M.	Sternoclavi- cular, finger foot, heels	4 yrs.	4 yrs.	1 week	10 wks.	60 mil.	2	—	14 days	Improved	Placebo. No fixation	Improvement. Returned to work
5	H. M. 26	M.	Sacroiliac, knee, an- kle, heel	3 yrs.	.....	1 wk.	10 days	400 mil.	2	—	7 days	Recovery	Rest in bed. Placebo	An acute case. Recovery rapid. Rest in bed no doubt a large factor
6	H. H. 28	M.	Knee	4 yrs.	.....	denied	4 wks.	400 mil.	6	—	34 days	Recovery	Rest in bed	Gonococcus isolated from seropurulent joint fluid. Repeated aspiration of fluid from joint. No ankylosis
7	J. T. 36	M.	Ankle, knee	14 yrs.	14 yrs.	2 wks.	4 wks.	400 mil.	6	4	30 days	Recovery	Placebo. Dispen- sary treatment	Gonococcus in uridral discharge. Cul- tures from knee joint sterile. Repeated aspiration of joint. No ankylosis
8	J. M. C. 36	M.	Ankle, wrist, knees, toe, heels	15 yrs.	4 yrs.	1 wk.	3 wks.	300 mil.	5	4	30 days	Recovery	Rest in bed. Placebo	Some improvement with 2 weeks' rest in bed before treatment. Marked reactions to earlier injections
9	A. C. 37	M.	Knee	.....	.....	denied?	2 wks.	250 mil.	7	1	30 days	Recovery	Rest in bed. Placebo	Gonococcus isolated from seropurulent joint fluid. Repeated aspiration of joint
10	E. G. 36	M.	Vertebrae, heels, peri- ostitis of tibia, and ilium	14 yrs.	6 yrs.	.....	7 mo.	400 mil.	20	7	3 mo.	Marked im- prove- ment	Allowed regular out- door exer- cise	A chronic case. Rapid improvement for first month. Slow but progressive im- provement till recovery practically com- plete. Massage of prostate

TABLE 1.—Continued.

Case No.	Age	Sex	Joints Involved	Original Infection	Pre-vious Arthritis	Date of Appar-ent Rec-urrence before Present Arthritis	Dura-tion of Present Arthritis before Treatment	Inocula-tions of Average Dose	Num-ber of Inoc-ulations	Clini-cal Reac-tions	Dura-tion of Treat-ment	Result	General Treatment	Remarks
11	O. H. 25	M.	Ankles, foot	5 yrs.	.....	5 wks.	10 days	250 mil.	6	2	34 days	Recovery	Light band- age. Rest in bed. Placibo. Tonics	An acute case. Two well-marked clinical reactions following injections
12	M. F. 38	M.	Ankles, knees, wrists	17 yrs.	3 yrs.	1 wk.	6 wks.	250 mil.	6	4	50 days	Marked improve- ment	Rest in bed. Tonics	A very extensive case, with profound clinical reaction after first injection of 500 mil. General condition poor, and con-valescence slow. Final result good
13	J. McN. 22	M.	Spine, ankle, knees	4 yrs.	.....	6 mo.	6 mo., exacer-bation 10 da.	100 mil. 300 mil.	5	1	26 days	Recovery	Rest in bed	An acute exacerbation. Organisms iso-lated from fluid from ankle and from blood. No bt. lesion demonstrable. Marked reaction after 2d injection
14	F. S. 24	M.	Shoulder, ankle, knees, heels	6 wks.	.....	.....	5 wks.	250 mil.	7	1	30 days	Recovery	Rest in bed 1 wk. pre- vious to treatment. Slight im-provement	A subacute case. No reaction after 2 in-jections of 100 million. A 3d injection of 300 mil., followed by a sharp rise in temperature 6 hrs. later, with some ex-acerbation of joint pain. Gonococci in prostatic fluid
15	J. M. 20	M.	Ankles, knees, me-tatarsals	5 wks.	.....	5 wks.	3 wks.	250 mil.	7	2	35 days	Recovery	Placibo No improve- ment with 2 wks. rest in bed previous to treatment	A subacute case. Patient in poor physical condition. Progress slow. Gonococci in urethral discharge
16	H. C. 35	M.	Knees, ankles, wrist, spine, heels	10 yrs.	7 yrs. 2 yrs.	.....	3 wks.	350 mil.	14	2 slight	3 mo.	No per- manent im-prove- ment	Partial rest. Tonics. Iodides without benefit	A chronic case; alcoholic; chronic nephri-tis with considerable edema; anemia; prostate large and tender; old history of syphilis; dacryocystitis 1 year; otitis media with onset of present arthritis. Several periods of improvement after in-jections of 500 million, followed by re-lapses. Gonococcus in prostatic fluid
17	C. K. 23	M.	Sacroiliac, knee, ankles	3 yrs.	3 yrs. 8 mo.	.....	3 wks.	150 mil. 500 mil.	4 4	0 1	20 days 20 days	Recovery	Rest in bed. Tonic	No response to treatment and no reaction during first period of 20 days using 100-million dose. When dose was raised to 500 million a slight reaction followed and improvement was steady. Gono-coccus isolated from knee-joint fluid

TABLE 1.—Continued.

Case No.	Age	Sex	Joints Involved	Original Infection	Pre-vious Arthritis	Date of Appar-ent Rec-ent Infection before Present Arthritis	Dura-tion of Present Arthritis before Treat-ment	Inocula-tions Average Dose	Num-ber of Inoc-ula-tions	Clini-cal Reac-tion	Dura-tion of Treat-ment	Result	General Treatment	Remarks
18	J. S. 40	M.	Ankles, spine, knee	3 yrs.	3 yrs. 1 yr.	Continued gleet	6 wks.	400 mil.	2	—	10 days	Some im-prove-ment	Rest in bed. Urotropin, pot. iodide, Bier's hy-peremia	Some improvement following injections. Injections discontinued on account of excess of other treatment
19	W. E. 23	M.	Knees, an-kles, cervi-cal spine	4 yrs.	.....	3 wks.	9 wks.	400 mil.	2	—	5 days 10 days	Marked im-prove-ment	Up and around ward	No improvement on rest in bed and salicylates for 6 weeks. Rapid improvement after 2 injections. Then passed from observation 10 days. When next seen had relapsed. Injections followed by ad marked improvement
20	J. W. 28	M.	Knee, ankle	6 mo.	.....	6 mo.	3 mo.	400 mil.	2	—	7 days	Improve-ment	Rest in bed and salicylates for 1 wk. prior to injections, with some im-provement	Joints improved rapidly during week fol-lowing injections
21	L. J. 25	M.	Ankle	7 wks.	.....	.....	5 wks.	50 mil.	3	—	11 days	Recovered	Partial rest	Was improving before treatment. Im-provement rapid after injections
22	T. W. 25	M.	Ankle	10 wks.	.....	.....	1 wk.	50 mil.	2	—	7 days	No im-prove-ment	Rest in bed	After injection the opsonic index rose from 0.0 to 3.2. Case passed from observa-tion without clinical improvement
23	L. S. 18	M.	Ankles, knee	5 wks.	.....	.....	3 wks.	50 mil.	2	—	12 days	Recovery	Partial rest	Patient improving when inoculations were begun. Opsonic index was 0.8, 1.0 on 2 days previous to 1st injection. After inoculation 0.6, 0.8, 0.7. Clinically progressively improving. After 2d in-jection index 1.1, 1.1
24	P. B. 33	M.	Spine, knee, hip, tem-poro-maxil-lary, heels, finger	4 yrs.	.....	6 wks.	3 wks.	400 mil.	3	—	14 days	Recovery	Patient up and around ward	Improving slowly up to time of beginning inoculations. Improvement more rapid after inoculations
25	N. N. 10	M.	Shoulder	3 wks.	.....	.....	12 days	50 mil.	2	—	8 days	Recovery	Ambulatory	Patient improving before inoculation. No apparent influence on already favorable course

TABLE 1.—Continued.

Case No.	Age	Sex	Joints Involved	Original Infection	Pre-vious Arthritis	Date of Apparent Infection before Present Arthritis	Duration of Present Arthritis before Treatment	Inoculations Average Dose	Number of Inoculations	Clinical Reactions	Duration of Treatment	Result	General Treatment	Remarks
26	P. M. 22	M.	Sternoclavicular, heel spine, knees, sacroiliac	4 mo.	.....	.....	10 wks.	300 mil.	3	1 slight	2 wks.	Recovery	Rest in bed	A mild but chronic case. A slight reaction followed first inoculation of 300 mil. Recovery apparently hastened by treatment.
27	C. W. 25	M.	Knees, ankle, heels, spine	4 mo.	.....	.....	2 mo.	500 mil.	6	3	1 mo.	Rapid improvement	Rest in bed for 2 wks. Then ambulatory treatment	Patient growing steadily worse at time was begun. Moderate reactions were obtained after the first two injections of 500 million cocc. Effusions in knee joints were aspirated as they recurred. The gonococcus was isolated from the joint fluid. Improvement was rapid after 2 injections and the patient returned to work in 1 month, though there was still tenderness in one foot.
28	A. L. 21	M.	Foot, wrist, heels, toes	6 wks.	.....	.....	4 wks.	400 mil.	8	2	5 wks.	Steady improvement	Ambulatory. Placebo	The patient was incapacitated for work as he was in laundry by arthritis in right wrist. No improvement for 3 wks. prior to treatment. Returned to work in 16 days after beginning treatment (4 injections). Injections continued with progressive improvement. Gonococci in prostatic secretion. Massage of prostate on 3 occasions. Two reactions after injections. Later doses of 500 mil. gave no reaction.
29	J. D. 22	M.	Ankles, knees, sacroiliac, finger	4 wks.	.....	.....	1 wk.	500 mil.	12	2	2 mo.	Improvement	Rest in bed, sod solution, late gr. xv; sod benzoate, gr. v; q. i. d. for 2 wks.	An acute case, with complicating pericarditis, pleuritis, and probable endocarditis. Gonococci in urethral discharge and isolated in culture from joint on 2 occasions. Blood cultures negative. Recovery. No apparent benefit from injections, though 2 reactions occurred after injections, with rise in temperature

TABLE 1.—Continued.

Case No.	Age	Sex	Joints Involved	Original Infection	Pre-vious Arthritis	Date of Apparent Infection before Present Arthritis	Duration of Present Arthritis before Treatment	Inoculations Average Dose	Number of Inoculations	Clinical Reactions	Duration of Treatment	Result	General Treatment	Remarks
30	M. N. 24	M.	Knees, ankle, toe, metatarsals, heels	2 yrs.	2 yrs.	?	10 wks.	500 mil. 200 mil.	4 11	1 slight	16 days	Improvement	Partial rest. Chloroform, gr. v, q. i. d.	Painful heels for 2 yrs. since initial arthritis. Acute exacerbation of arthritis for 7 wks. with urethritis. Some improvement with rest in bed for 10 days previous to treatment. Arthritis subsided rapidly after injections. On leaving hospital there was slight tenderness of one knee, and tenderness in heels persisted. The patient returned at intervals of 1 wk. for 10 weeks, receiving an average dose of 200 mil. At the end of this time, there was no demonstrable tenderness in the heels and the joints all appeared normal.
31	C. A. 31	M.	Heels, back, acromio-clavicular, periositis of patella and tibia	6 yrs.	.....	.....	3 yrs. at intervals	300 mil.	..	—	3 mos.	Improvement	Ambulatory	A chronic case of 3 yrs. standing. Partial disability from tender heels and pain in back. No improvement on medical treatment. No improvement on local treatments, etc. Prostate large, tender. Many typical gonococci in prostatic fluid. After each of early injections (200 mil.) pain gradually subsided for 24 hrs. Slow improvement with injections at intervals of 4-5 days. Prostatic massage. After 1 month distinctly better. After 3 months, though not cured, decidedly better than for 3 years previously.

## NOTE ON THE NATURE OF OPSONIC IMMUNITY.\*

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STIMULATED by the work of Sir Almroth E. Wright, various observers have recently investigated the question of phagocytosis in connection with the theory of opsonic immunity, and have obtained the following rather generally accepted results:

1. Active phagocytosis, *in vitro*, of many of the common forms of bacteria is dependent upon the action of blood serum or similar body fluids, and does not take place when these fluids are replaced by physiological salt solution.

2. Almost no phagocytosis occurs in serum which has been exposed to a temperature of 60° C. for 10 to 30 minutes.

3. Serum is deopsonized, i. e., it is inactivated, by digestion with a bacterial emulsion with subsequent removal of the bacteria by centrifugalization.

With these results as a basis, we have carried out some of the fundamental experiments in phagocytosis. As regards the relation of opsonins to immunity, it is of importance to decide, first of all, whether or not phagocytosis, as it occurs normally, is an inherent property of the leucocytes and independent of any action of the body fluids. In view of the differences sometimes found in the serum and plasma of the same individual, for example as regards the enzyme content,<sup>1</sup> it would be of both clinical and scientific interest to determine whether opsonins are present in the plasma and whether the breaking down of the formed elements during the clotting of the blood has any influence on phagocytosis.

Wright and Douglas<sup>2</sup> found the same opsonic content both in plasma, obtained by decalcifying blood, and in the corresponding serum. This method of obtaining plasma is of questionable service in deciding the rôle of the formed elements, since mere precipitation of the calcium salts does not entirely prevent the breaking down of the cells in the shed blood. To avoid this difficulty, advantage was taken of the fact that blood, removed from the body in resected

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bloodvessels, will stand for long periods without clotting.<sup>3</sup> It should be possible then to resect a blood vessel, precipitate the corpuscles by centrifugalization, and pipette off the supernatant plasma without bringing it in contact with a cut surface. For studying the rôle of the cellular elements, this principle was employed in the following way.

Rabbits were used, and in preparing them no anesthetic was administered, in order to avoid any possible complications arising from its anti-phagocytic action. In order that sensation might be abolished the animals were pithed between the occipital bone and the atlas. A tracheal cannula was at once inserted and artificial respiration supplied. The heart was found to be the most suitable part of the vascular system for resection as its muscular wall afforded a substantial protection to the intima during the subsequent manipulations. Plasma obtained in this manner could not be used directly for phagocytic work, since the addition of the leucocytic emulsion would furnish the only factor lacking for coagulation. The presence of fibrin after the addition of the bacterial emulsion is objectionable since the bacteria collect in its meshes and escape the action of the leucocytes, thereby giving entirely misleading results. In order to avoid this difficulty the phagocytic preparations were not made until after spontaneous coagulation had occurred, which took place after an hour's time at room temperature. Preparations were then made in the ordinary manner.

We have followed very closely the usual opsonic technique. *Staphylococcus* cultures were obtained from two different sources but both were of rather low virulence. The *staphylococcus* emulsions were always prepared from a one-day culture on agar at 37° C.

In the preparation of the leucocytic emulsion, two washings were used. One-half to one c.c. of blood was collected in about 10 c.c. of a solution composed of 1 per cent sodium citrate and 0.85 per cent sodium chloride which was contained in an ordinary centrifuge tube. After centrifugalization and without removing the supernatant fluid, approximately 0.2 c.c. of the blood-cream, on the surface of the corpuscles, was pipetted off and rewashed with about 10 c.c. of 0.85 per cent sodium chloride solution. This procedure gave emulsions rich in leucocytes and practically devoid of phagocytic power in the absence of serum. Except where otherwise stated, the leucocytes and serum were always obtained from normal human blood. For isotonic salt solution, 0.85 per cent sodium chloride was employed.

The preparations were put up in pipettes. Equal volumes were used of each of the different fluids entering into the preparation, for example: one volume of leuco

cytic emulsion, one volume of bacterial emulsion, and one volume of serum. Unless otherwise stated, the preparations were incubated for 30 minutes at 37° C. Smears were made as advised by Wright and were stained by Hasting's modification of Jenner's stain. In counting the preparations, we have depended upon the results obtained from enumerating the bacteria in 50 consecutive polymorphonuclear-neutrophile cells. Owing to the unavoidable errors associated with the method, no emphasis has been placed on slight quantitative variations.<sup>4</sup> The experiments have been confined to preparations giving practically qualitative results, i. e., either active or minimal phagocytosis.

In a typical case a count of 50 leucocytes showed a total of 653 as compared with 1,067 bacteria for a preparation with serum of the same animal collected in the ordinary manner. As a control, a third preparation with salt solution gave a count of 67 bacteria per 50 leucocytes. Although this process eliminated a very high percentage of the cellular elements, a very definite diminution in phagocytosis was not obtained. When leucocytes are tested directly, *in vivo*, for example, by the injection of typhoid bacteria into the circulation,<sup>5</sup> active phagocytosis at once takes place. If inflammation or fibrin-formation is excluded, this can be taken as proof of the activity of the plasma, except for the possibility that some property of the leucocytes is lost in their treatment outside of the body.

Other experiments also indicate that opsonin does not arise during the breaking down of the formed elements of the blood. Neumann<sup>6</sup> found that extracts of leucocytes do not favor phagocytosis. I have obtained a similar result for platelets. For their preparation, the method described by Cole was employed.<sup>7</sup> Normal human blood was collected in a solution containing 1.5 per cent sodium citrate and 0.85 per cent sodium chloride. The corpuscles were then separated by fractional centrifugalization. The platelets readily remained in suspension, coming down principally in the later fractions. After recentrifugalizing three or four times with salt solution, they were obtained practically free from other cells. An oese of platelets was digested in salt solution at 37° C. for two hours. Upon adding this mixture to an emulsion of leucocytes and staphylococci, no more phagocytosis resulted than in the control preparation with salt solution.

Although absolute conclusions are not justified, it seems probable that active phagocytosis occurs both in plasma and in serum.\*

\* In a preliminary communication, Briscoe<sup>8</sup> expresses a different opinion. His final results have not yet been reported.

## EVIDENCE OF OPSONIC ACTION.

Wright<sup>1,2</sup> considers that the opsonic action of the serum consists in a specific modification of the bacteria ingested. The following experiment is the one originally devised for the demonstration of opsonic action:

Three preparations are necessary.

A. A mixture of equal volumes of bacterial emulsion, of washed leucocytes, and of serum, when incubated, gives pronounced phagocytosis.

B. Another preparation is made containing a mixture of bacterial emulsion and serum. This is first digested for 15 minutes at 37° C., and then heated at 60° C. for 10 minutes or longer. Now when leucocytes are added to this, pronounced phagocytosis also occurs.

C. As a control, a third preparation is necessary. This is similar to the second, except that the mixture of serum and bacterial emulsion is heated immediately to 60° C. without the preliminary digestion. When subsequently incubated with leucocytes, only a minimal phagocytosis occurs.

In this experiment there are two points of especial importance: (1) Some phagocytosis *does* occur in the control preparation (Prep. C); (2) The amount of phagocytosis after digesting and heating the bacterial emulsion (Prep. B) is somewhat *diminished* as compared with the untreated emulsion (Prep. A). These differences, in the case of cocci at least, are so definite that they are practically qualitative and are well outside the limits of experimental error.

Bulloch and Atkin<sup>3</sup> have confirmed these observations. We have also repeated this experiment with the following results:

Mixtures	Bacteria per 50 leucocytes		
	I	II	III
A) Staphylococcus emulsion + serum + washed leucocytes. . . . .	680	726	632
B) Staphylococcus emulsion + serum, digested 15 minutes at 37° C. and heated 30 minutes at 60° C. + washed leucocytes. . . . .	529	699	490
C) Staphylococcus emulsion + serum, mixed and heated at once for 30 minutes at 60° C. + washed leucocytes. . . . .	208	192	114

In considering the possibility of the coexistence of opsonins and stimulins it has been noted<sup>2</sup> that the phagocytosis in the case of the digested and heated bacteria (Prep. B) is slightly diminished. The constancy of the diminution in the same direction might suggest

that some stimulin action had been destroyed during the heating. There is a simpler explanation, however, for we found that the clumping of the bacteria during the heating diminished the numbers of free cocci available for phagocytosis.

Hektoen and Ruediger<sup>11</sup> introduced the following variation in the preceding experiment. In the second preparation, the bacterial emulsion and serum, immediately after digestion at 37° C., were separated by centrifugalization and the bacteria were then heated to 60° C. in salt solution instead of in serum. After this treatment, the bacteria were neither phagocytized when suspended in salt solution nor even in the presence of active serum. They consider that these results contradict Wright's observations and they also draw interesting conclusions from them in regard to the structure of opsonins.

In the repetition of this modification, sensitized bacteria were obtained by digesting equal volumes of serum and staphylococcus emulsion (about 0.2 to 0.3 c.c. each) at 37° C., for 30 minutes. This mixture was then diluted with about 10 c.c. normal salt solution and the bacteria were collected by centrifugalization. An oese of the bacterial precipitate was emulsified in salt solution.

Mixtures	Bacteria per 50 leucocytes	
	Experiments I	Experiments II
A) Sensitized staphylococci + washed leucocytes + salt solution. . . . .	258	476
B) Sensitized staphylococci, heated 30 minutes at 60° C. + washed leucocytes + salt solution. . . . .	47	91
C) Sensitized staphylococci, heated for 30 minutes at 60° C. + washed leucocytes + serum. . . . .	291	379

The necessary controls show that the treatment by heat in the second preparation is responsible for the loss of phagocytic action. These results agree with Hektoen and Ruediger's except that in the third preparation we were not able to demonstrate any change in the bacteria preventing the action of the leucocytes in the presence of active serum. This difference might perhaps be accounted for by the fact that I worked with staphylococci whereas their results are given for streptococci.

The interpretation of these two experiments presents some difficulties. It is not altogether clear whether or not one can have active phagocytosis of unopsonized bacteria, in a case where serum is necessary for phagocytosis. The problem is rendered perhaps a little simpler by making a slight modification in Hektoen and Ruc-

diger's experiment. Since sensitized bacteria are not phagocyted after heating, a similar result might be obtained directly, using dilute serum, instead of first sensitizing the bacteria with serum which is later to be diluted by washing.

Preparations were made as follows, using a bacterial emulsion of only moderate strength:

Mixtures	Bacteria per 50 leucocytes Experiments	
	I	II
A) Staphylococcus emulsion + serum (1-10 dilution) + washed leucocytes.....	221	447
B) Staphylococcus emulsion + serum (1-10 dilution) digested 20 minutes at 37° C. and heated 30 minutes at 60° C. + washed leucocytes.....	51	122
C) Staphylococcus emulsion + serum (1-10 dilution) mixed and heated at once for 30 minutes at 60° C. + washed leucocytes	18	30

Here we have active phagocytosis although the control preparations show no evidence of any alteration in the bacteria.

Another fundamental experiment for demonstrating the rôle of the serum in phagocytosis is as follows: Leucocytes when washed by centrifugalization with normal salt solution show no evidence, as far as phagocytosis is concerned, of any reaction with or any fixation of any part of the plasma, although they have been bathed in it continuously. On the other hand, bacteria, after digestion with normal serum, are still phagocyted actively after fairly complete removal of the serum by washing.<sup>11</sup> This is considered as proof of opsonic action by many workers. But the experiment upon which this statement is based does show that considerable diminution in phagocytosis after washing occurs. This diminution is attributed to a dilution of the bacterial emulsion during manipulation. Sufficient controls, though, were not recorded for discriminating between a loss of opsonin by washing and a dilution of the emulsion by loss of bacteria.

As compared with leucocytes, the mechanical difficulties are somewhat greater in applying this experiment to bacteria. In order to effect thorough washing, we used a bacterial emulsion and serum in equal quantities (about 0.2 to 0.3 c.c. each). After digestion at 37° C. for 30 minutes, a sample was reserved and the mixture diluted with 11 c.c. of normal salt solution contained in an ordinary centrifuge tube. Centrifugalization for 45 to 60 minutes with an electrically driven centrifuge (2,000 revolutions per minute) was found sufficient to throw down the bacteria. During the precipitation,

the bacteria became extensively massed together and in order to expose them satisfactorily to the washing fluid, it was necessary to emulsify them thoroughly after each precipitation. At the end of each washing the emulsion was restored to its original concentration and a small sample reserved. Two preparations were made from each sample. One contained equal volumes of washed leucocytes, bacterial emulsion, and salt solution. The second was a duplicate of this except that one volume of serum was substituted for the volume of salt solution. Such controls are necessary and sufficient to locate the factors which may be responsible for any change in phagocytosis.

The experimental data are as follows:

TABLE I.  
EFFECT OF WASHING BACTERIA AFTER TREATMENT WITH NORMAL SERUM.

NUMBER OF WASHINGS	STAPHYLOCOCCUS PYOGENES AUREUS		BACILLUS TUBERCULOSIS		STAPHYLOCOCCUS PYOGENES AUREUS		APPROXIMATE DILUTION
	Serum	Salt Sol.	Serum	Salt Sol.	Serum	Salt Sol.	
Without washing .....	1.018	738	220	102	706	517	1-1
First washing .....	0.95	330	210	105	533	275	1-30
Second washing .....	1.290	40	217	104	003	02	1-500
Third washing .....	...	...	145	37	...	...	1-15,000
Control with salt solution ..	110		32		11		

The figures given express the number of bacteria per 50 leucocytes. Although the preparations with salt solution need not necessarily be equal to the corresponding serum preparations, yet there should be practically no diminution with the successive washings unless some opsonin has been lost. The approximate dilutions are stated without regard to the precipitate of bacteria present and are intended merely to give some idea of the amount of washing employed. In comparing leucocytes and bacteria with regard to the amount of washing necessary to free them from serum, one must consider the theory of washing precipitates and the phenomena of adsorption.<sup>12</sup> The extent of surface condensation of fluids on precipitates depends partly upon the area of exposed precipitate. In proportion to their volume, then, the bacteria offer a larger surface area than the leucocytes for the retention of serum since the area of a sphere grows proportionally smaller as its volume increases. Thus the area of a leucocyte would be approximately 10 times less than the surface area

of an equal volume of staphylococci on the basis of an average diameter of 10 microns for a leucocyte and 1 micron for a staphylococcus.

The results of this experiment do not contradict any previously reported work. The bacteria at the end of the first washing correspond entirely, in their properties, to Hektoen and Ruediger's sensitized bacteria and we have used them as such with satisfactory results. The washing experiments of Neufeld and Rimpau<sup>13</sup> do not concern us in this connection, since they worked with immune sera. They used only one washing, however, and since they do not state the volumes of fluid used, it is not altogether certain that the bacteria were finally freed from serum. Bächer,<sup>14</sup> working on this problem, came to the same conclusions which Hektoen reached. A diminution in phagocytosis was obtained in some cases, but he also attributes this to a loss of bacteria from mechanical causes. Recently, Sleeswijk<sup>15</sup> considers that he has demonstrated opsonic action in the case of anthrax bacilli and frog serum. This same principle was employed, but since only one washing was used, it is not unlikely that sensitized bacteria were obtained, corresponding to the bacteria usually obtained at the end of the first washing. The experiment is open to further objections. Other observers,<sup>16</sup> in order to obtain satisfactory preparations with anthrax, have found it necessary to work with spores on account of the long chains which the bacteria form. There is even some dispute as to whether serum is necessary for the phagocytosis of anthrax bacteria.<sup>17-18</sup> Sleeswijk reports a strong agglutinating action of the frog serum upon the anthrax bacilli. This must have increased the difficulty of thoroughly exposing these agglutinated clumps to the washing fluid. These clumps must also have given further difficulty in the study of their phagocytosis. Dean<sup>19</sup> found that when bacteria, which have been supersaturated with serum, are suspended in salt solution, the opsonin diffuses into the surrounding medium. Centanni<sup>34</sup> has reported that the opsonic reaction between serum and pneumococci can readily be destroyed by washing. He also makes the important statement that after several repetitions of this process, the pneumococci are no longer phagocytosed even in the presence of active serum.

This experiment does not permit us to draw final conclusions.

The possibility of opsonic action is neither proven nor disproven. One might suppose, for instance, that there is a reaction between the bacteria and the serum, perhaps a chemical reaction, which is a reversible one. Then if its direction depended upon the dilution employed, any opsonic effect might readily be destroyed by washing. These results have, however, a few points of practical importance in connection with such experiments as the following:

1. Sensitization methods have been developed especially by Hektoen and they are particularly applicable in many experiments, for example, in distinguishing between anti-opsonic and anti-phagocytic substances. If no phagocytosis resulted in a preparation where the bacteria were presumably sensitized, control tests would be necessary to differentiate between an inactive fluid and an active serum which might have been completely removed by washing.

2. If serum has no stimulating effect upon leucocytes, one might expect that after sensitizing bacteria, the serum could be discarded or that if it were injected along with the bacteria it would have no protective effect upon the inoculated animal. Bordet and Gengou's<sup>20</sup> study of the comparison of the injection of (a) untreated bacteria, of (b) sensitized bacteria, and of (c) bacteria mixed with serum, showed that the sensitized and non-sensitized bacteria killed the animals into which they were injected while the animal which received the simultaneous injection of serum survived.

#### DEOPSONIZING EXPERIMENTS.

The technique of the deopsonizing experiments is briefly as follows: Equal volumes of a heavy bacterial emulsion and serum are incubated at 37°C. ° for 15 to 30 minutes. The mixture is then centrifugalized until the bacteria are precipitated and the supernatant fluid is free from organisms. The bacterial sediment is discarded and the serum, when tested with a fresh emulsion of bacteria in the ordinary manner, is found to have lost most of its opsonic power.

In the case of heated immune sera and the tubercle bacillus, Wright and Reid<sup>21</sup> consider this single procedure as proof of opsonic action. Control tests are necessary for the final interpretation of such an experiment. Whatever may be the probable explanation, at least two possibilities demand consideration: (1) either an injurious



substance may have been introduced, or (2) a substance favoring phagocytosis may have been removed.\*

The first of the two possibilities suggested might have an important bearing upon specificity determinations, but as regards the question of the chemical or physical aspects of the opsonic reaction, only the second one is of especial interest. Either the opsonin may combine chemically with the bacteria, or it may be removed mechanically from the serum by the bacterial emulsion, somewhat as enzymes are carried out of solution by foreign bodies. In support of this mechanical explanation, it is found that serum is deopsonized by bacteria at  $0^{\circ}\text{C}$ .,<sup>23</sup> the low temperature itself not being responsible for any deleterious action on the serum. Bulloch and Atkin<sup>10</sup> consider that the bacteria themselves are opsonized. To test this point, we applied the original heating experiment of Wright with the following results:

Mixtures	Bacteria per 50 leucocytes	
	Experiments I	II
A) Staphylococcus emulsion + serum, digested 15 minutes at $37^{\circ}\text{C}$ . and heated 30 minutes at $60^{\circ}\text{C}$ . + washed leucocytes. . . . .	490	699
B) Staphylococcus emulsion + serum, digested 15 minutes at $0^{\circ}\text{C}$ . and heated 30 minutes at $60^{\circ}\text{C}$ . + washed leucocytes. . . . .	159	60
C) Staphylococcus emulsion + serum, heated at once for 30 minutes at $60^{\circ}\text{C}$ . + washed leucocytes. . . . .	114	192

In preparation *B*, both the serum and the bacterial emulsion were cooled to  $0^{\circ}\text{C}$ . before mixing.

Thus, although we may have serum deopsonized at  $0^{\circ}\text{C}$ ., it cannot be shown that the bacteria themselves are opsonized. Cowie and Chapin<sup>24</sup> have carried out a variety of deopsonizing experiments in working upon the amboceptor-complement structure of normal opsonins. They find different types of deopsonized serum according to whether the amboceptor or the complement is destroyed. It might be of importance to determine what type of inactivation is effected by deopsonizing with carbon particles.

As further evidence of the possibility of the mechanical removal of opsonin, Bächer,<sup>14</sup> and also Simon, working with Lamar and Bispham,<sup>25</sup> found that serum is deopsonized by foreign substances,

\* In this connection Ledingham<sup>22</sup> finds that "Heated normal serum through which tubercle bacilli have been passed exerts a marked inhibitory action . . . on the opsonin of fresh normal serum." He suggests a slightly different explanation, namely, the action of opsonic amboceptors with the phenomena of deviation of the complement by free receptors thrown off from the bacteria into the heated serum.

such as carmine, charcoal, and filter paper. Charcoal is known to absorb a variety of substances, such as gases, coloring matter, proteids, and also enzymes.<sup>26</sup> In testing the specificity of the deopsonizing action, we compared the activity of normal serum and of serum digested with carbon at 37° C. for 20 minutes with subsequent removal of the carbon by centrifugalization.

Mixtures	Bacteria per 50 leucocytes	
	Experiments I	II
A) Washed leucocytes + staphylococcus emulsion + serum.....	591	869
B) Washed leucocytes + staphylococcus emulsion + treated serum...	167	220

#### EVIDENCE OF THE ACTION OF SERUM UPON LEUCOCYTES.

Turning to the possibility of an action of the serum upon the leucocytes, it is important to remember that the theory of stimulin action does *not* necessarily involve any chemical reaction.<sup>27</sup> Indeed one would expect the leucocytes to have come into a state of chemical equilibrium with the surrounding plasma. It is altogether possible that the serum, though necessary for the phagocytosis, reacts, neither with the leucocytes nor with the bacteria, but it may exert its influence in a catalytic manner.

In considering whether serum has any direct effect upon leucocytes, the variable factors in the problem may be reduced by substituting a chemically inert body for the bacteria. This limits one then to the effect of the serum upon the leucocytes.

In applying this principle, Wright and Douglas<sup>2</sup> came to the conclusion that serum probably favored the phagocytosis both of carmine and of India-ink particles. To secure an inert body in a suitable condition for phagocytosis, we used chemically pure carbon black, suspended in normal salt solution. In emulsifying it, the smaller particles were massed together upon the addition of liquid. A satisfactory separation, suitable for subsequent enumeration, was obtained by the same process usually recommended for the preparation of tubercle bacilli emulsions. The most satisfactory preparations were secured by using dilute emulsions and incubating for comparatively long periods. This avoided, to a large extent, the superimposing of leucocytes and carbon which could be seen in working with thick emulsions when smears were made immediately

upon mixing, without waiting for an incubation period. The following preparations were incubated for 45 minutes:

Mixtures	Carbon particles per 50 leucocytes		
	Experiments		
	I	II	III
A) Washed leucocytes + carbon emulsion + serum.....	411	215	75
B) Washed leucocytes + carbon emulsion + salt solution.....	76	53	10

These data show that the presence of serum was necessary for the phagocytosis of carbon particles. As to whether the serum is inactivated by heat for carbon particles, a dilute emulsion gave the following results:

Mixtures	Carbon particles per 50 leucocytes	
	Experiments	
	I	II
A) Washed leucocytes + carbon emulsion + serum.....	411	215
B) Washed leucocytes + carbon emulsion + serum, heated for 30 minutes at 60° C.....	67	99

With a concentrated carbon emulsion the difference was less striking:

Mixtures	Carbon particles per 50 leucocytes
A) Washed leucocytes + carbon emulsion + serum.....	347
B) Washed leucocytes + carbon emulsion + serum, heated for 30 minutes at 60° C.....	259

As regards the extent of inactivation of serum by heat for bacteria, apparently considerable variation occurs according to the concentrations employed.

In testing whether or not the heating experiment, as performed for the demonstration of opsonins, could be carried out with carbon particles, I obtained a uniformly negative result. An especially troublesome feature was the clumping of the carbon particles during the heating. I was, therefore, unable to repeat all of the opsonic experiments with an inert body such as the carbon particles employed. The experiments do show that the presence of serum does exert a favorable influence directly upon the leucocytes.

#### EFFECT OF THE SERUM UPON THE MOTILITY OF THE LEUCOCYTES.

As further evidence of the effect of serum upon leucocytes, it is found that when a drop of freshly drawn blood is collected in salt solution and examined upon a warm stage at 37° C., many of the leucocytes show definite amoeboid locomotion. In contrast to this, the leucocytes, when washed by centrifugalization for the preparation

of the emulsion in salt solution, show at most only an occasional pseudopod and are practically devoid of locomotion. Upon the addition of serum, careful examination of hanging drops upon the warm stage shows that they have regained both their intrinsic movements and to a limited extent their power of locomotion. In testing the motility in serum, heated for 30 minutes at 60° C., only a doubtful result was obtained. Sleeswijk<sup>15</sup> found that the leucocytes were non-motile when suspended with anthrax bacteria in salt solution and also practically non-motile when suspended with anthrax bacteria in heated serum. No observations were recorded for preparations with active serum.

#### CLUMPING ACTION OF THE SERUM.

The examination of opsonic preparations in hanging drops raises the question, Do the leucocytes, *in vitro*, move actively from place to place in search of bacteria, or do they merely engulf the neighboring organisms? Rosenow<sup>28</sup> found that after leucocytes have been killed by heat, the presence of opsonic serum causes the bacteria to be clumped around the leucocytes. Our results tend to support this conclusion.

An emulsion of leucocytes, after exposure to a temperature of 45° C. for 10 minutes, showed no motility whatever. (Rosenow has found that longer periods are necessary to destroy all phagocytic power.) Three ordinary opsonic preparations were then made with the heated emulsion, using (1) normal serum, (2) serum heated for 30 minutes at 60° C., and (3) salt solution. A hanging-drop mount was then made from each preparation. In the case of the normal serum, the majority of the staphylococci remained free, but a few were grouped about the leucocytes. In both the heated serum and the salt solution, the cocci showed no tendency to group about the leucocytes. At the end of 45 minutes, the hanging drops were allowed to dry, and after staining with Hasting's stain, the bacteria lying in contact with the leucocytes were counted.

Mixtures	Bacteria per 50 leucocytes	
	Experiments I	II
A) Heated leucocytes + staphylococcus emulsion + serum.....	296	244
B) Heated leucocytes + staphylococcus emulsion + heated serum....	27	60
C) Heated leucocytes + staphylococcus emulsion + salt solution....	46	20

Although opsonins are held to be distinct from other immune

bodies, it is interesting, in considering this heterologous agglutination, that both high opsonic and high agglutinating serum is obtained in typhoid-immune animals<sup>29, 30, 31</sup> and also that in other diseases, such as staphylococcus infections, very low values are obtained for both agglutinins and opsonins.

#### ACTION OF THE SALTS IN PHAGOCYTOSIS.

For the demonstration of definite substances, in serum, essential for phagocytosis, but presumably without action on the bacteria, Dr. Cole suggested a study of the salts. The work of Bordet<sup>32</sup>, and Joos<sup>33</sup>, showing the necessity of salts for agglutination, indicates a similar possibility in phagocytosis.

In the preparation of salt-free phagocytic mixtures, the tonicity of the fluids was maintained with a 5.5 per cent solution of saccharose (Kahlbaum's). To prepare the leucocytic emulsion, 1 c.c. of blood was collected in 2 c.c. of saccharose solution containing 1 per cent of sodium citrate. This mixture of 3 c.c. was washed with 9 c.c. of isotonic saccharose solution and after collecting the leucocytes (about 0.3 c.c. volume), they were washed once more with 10 c.c. of saccharose solution.

In the preparation of the staphylococci and the serum, about 0.25 c.c. of staphylococcus emulsion in saccharose solution was digested with an equal volume of serum for 30 minutes at 36° C. The mixture was then diluted with 10 c.c. of saccharose solution, the bacteria were collected by centrifugalization, and an oese of the bacterial precipitate was re-emulsified in saccharose solution.

Mixtures	Bacteria per 50 leucocytes I
A) Washed leucocytes + sensitized bacteria + saccharose solution.....	47
B) Washed leucocytes + sensitized bacteria + salt solution.....	258
C) Washed leucocytes + sensitized bacteria + serum.....	291

In a repetition of this experiment, the staphylococci were emulsified in distilled water, digested with serum, and then washed with distilled water. An oese of the bacterial precipitate was emulsified as before, in saccharose solution.

Mixtures	Bacteria per 50 leucocytes II
A) Washed leucocytes + sensitized bacteria + saccharose solution.....	43
B) Washed leucocytes + sensitized bacteria + salt solution.....	235

## SUMMARY.

1. Any reaction, favoring phagocytosis, which may take place between *Staph. pyogenes aureus* and normal serum at 37° C., can be broken up by thorough washing.

2. Active phagocytosis of staphylococci is possible under conditions in which no opsonic action can be demonstrated, either by heating or by washing experiments.

3. One of the essential factors for phagocytosis, namely, the presence of salts, presumably does not owe its effect to an alteration of the bacteria.

4. Evidence of the action of the serum upon the leucocytes is shown by: (a) Their loss of motility in salt solution emulsions, and (b) The occurrence of phagocytosis of chemically inert bodies only in the presence of serum.

5. Further possibility of the mechanism of the action of the serum *in vitro* is seen in the clumping of the leucocytes and the bacteria.

Observers are not agreed as to what constitutes the proof of opsonic action. In the complex of substances present in the various bacteria and sera, one cannot expect that a reaction between them would never take place.

The experimental data at hand do not yet justify the conclusions for normal serum and staphylococci, a very typical case, that: (1) The predominating and essential factor in the conditions favoring phagocytosis is an alteration of the bacteria, nor that, (2) The serum by its presence is not of considerable importance in favoring the activity of the leucocytes.

At this opportunity, I wish to express my thanks to Dr. R. I. Cole, under whose direction this work has been carried on in the biological laboratory of the medical clinic.

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## STUDIES UPON THE AMEBAE IN THE INTESTINE OF MAN.\*†

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IN 1903 appeared Schaudinn's<sup>1</sup> conclusions regarding the amebae infesting the human intestine. As the result of his researches he clearly differentiated and described two species of amebae parasitic in man, one, pathogenic, producing the lesions of amebic dysentery, which he called *Entamoeba histolytica*; the other, non-pathogenic, to which he gave the name, *Entamoeba coli*.

At the time Schaudinn's publication appeared the writer was in charge of the laboratories of the U. S. Army General Hospital in San Francisco, Cal., where hundreds of cases of amebic dysentery had been under observation and treatment, and where there was always abundant material for the study of amebae. After over a year of constant work upon the subject I published the results of my investigations,<sup>2</sup> which largely confirmed those of Schaudinn, and which have been in turn confirmed by numerous independent observers. My conclusions at that time were briefly as follows:

The intestine of man may be infested with two species of amebae, one, pathogenic, the other, non-pathogenic; the non-pathogenic ameba (*Entamoeba coli*) was found in 65 per cent of healthy individuals examined, and in 50 per cent of those suffering from diseases other than dysentery; the two species may be distinguished in both fresh and stained preparations; they differ in their method of reproduction; the pathogenic ameba (*Entamoeba histolytica*), whether fed in milk or injected into the rectum produces in kittens the lesions of dysentery as seen in man; the non-pathogenic ameba, whether fed in milk or injected into the rectum, never produces in kittens any lesions whatever; neither feeding experiments nor rectal injections of fecal material, or the bacteria occurring in such material, produce in kittens

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any of the lesions of amebic dysentery, unless *Entamoeba histolytica* be present.

While at the present time Schaudinn's classification of the intestinal amebae in man has been accepted by such zoölogists as Stiles<sup>3</sup> and Minchin,<sup>4</sup> and by almost every investigator of, and writer upon, amebic dysentery, there are some authors who still hesitate to accept the division of the amebae into a pathogenic and non-pathogenic species, preferring to believe that the pathogenicity of these organisms, depends upon their environment or that, as yet, we have not sufficient evidence to warrant us in differentiating species.

In view of this fact any contribution upon the amebae in man, based upon the results of personal observation and careful study, should prove of some value in the elucidation of this question, and it is my purpose, in this contribution, to give in detail the results obtained from the study of the amebae observed in 1,579 cases of amebic dysentery, and from the study of the amebae observed in the feces in health and in diseases other than dysentery, together with the evidence obtained by the experimental production of the disease in animals.

Those who have used cultural forms of amebae as an argument against species have apparently lost sight of the fact that all known protozoa that have been cultivated develop, in cultures, very different forms from those usually observed, as, for instance, in the case of the trypanosomes and the Leishman-Donovan bodies. To compare amebae derived from external sources and cultivated upon artificial media with *Entamoeba coli* and *Entamoeba histolytica* as observed in the feces, and by the results of such comparison to claim on morphological grounds, that it is impossible to differentiate these two species, is surely an unscientific deduction and one that cannot be accepted as fact. In this paper, therefore, the amebae are described as they appear in the feces or in the intestine, and no deductions are drawn from the morphology of cultivated organisms. I am firmly of the opinion that the evidence already at hand regarding the two species of amebae described by Schaudinn is amply sufficient to establish the truth of his observations, but if further evidence is needed, it would appear that the question could be definitely settled by the cultivation, side by side, upon the same media, of *Entamoeba coli* and *Entamoeba histolytica*, an obvious and apparently simple method which appears

to have been overlooked by Schaudinn's opponents, for nowhere in the literature can I find a record of such experiments, although all kinds of amebae have been cultivated from extraneous sources and compared to the species of amebae occurring in man. While *Entamoeba histolytica* appears to have been cultivated, no instance is on record of the cultivation of *Entamoeba coli*, or even an attempt to do so, under similar circumstances, and upon similar media. Personally I have found it impossible to cultivate *Entamoeba coli* upon the media used for other amebae.

However, it is obvious that if it were possible, the cultivation of the amebae found in health and those found in amebic dysentery, with the determination of the pathogenic action of such cultures upon susceptible animals, would settle for all time the question of the existence of pathogenic and non-pathogenic species, but until this has been accomplished, we must accept the accumulated evidence, both morphological and experimental in favor of Schaudinn's classification as sufficient to prove its truth.

Prior to the perusal of Schaudinn's paper I had undoubtedly confused the two species of amebae, regarding the harmless *Entamoeba coli* as an atypical form, or as a stage in the development of *Entamoeba histolytica*. Both species often occur together in the feces of cases of amebic dysentery, and this fact has been one of the principal stumbling-blocks in the identification of the two organisms. If they be studied separately, however, the variation in morphology is so marked as to render their differentiation a matter of but little difficulty in the vast majority of instances. The harmless *Entamoeba coli* is a very common parasite of man and this renders the study of the intestinal amebae of great importance, as it is undoubtedly true that many cases have been erroneously diagnosed as amebic dysentery which were in reality cases of diarrhea due to other causes but in which *Entamoeba coli* was present in the feces and was mistaken for *Entamoeba histolytica*. This fact may account for the wonderfully successful results in the treatment of amebic dysentery claimed by some practitioners in the tropics.

As would be expected, I have seen certain cases infested with *Entamoeba coli* develop true dysenteric symptoms, with *Entamoeba histolytica* in the feces, but in an amebae infected country, such as the

Philippines, it would be strange indeed if certain of the individuals showing the harmless ameba in their feces did not, sooner or later, become infected with the pathogenic variety, especially when, as I shall show, 72 per cent of white men examined harbored the harmless ameba. Those opposed to Schaudinn's classification have tried to use such cases as an argument in favor of the unity of all intestinal amebae, but with the chances of infection so great as they are in the tropics, an argument based upon these cases has but little, if any, scientific value. It should also be remembered that a man might be suffering at the time of examination with amebic dysentery, yet only *Entamoeba coli* be found in his feces, and if only one examination were made the true condition would not be discovered. However, a few isolated cases of this kind, so easily explained, have no weight as a scientific argument against the truth of Schaudinn's classification of the intestinal amebae in man.

#### HISTORICAL.

Probably the first investigator to observe amebae in the stools of man was Lambl<sup>5</sup>, of Prague, who, in 1860, described organisms occurring in the feces of a child suffering from diarrhea which he interpreted as amebae, although they may have been stages in the development of one of the intestinal flagellates. In 1870, Lewis and Cunningham<sup>6</sup> found amebae in the feces of nearly 20 per cent of cholera patients studied in India, and again, in 1881, in cholera stools and in those of patients suffering from other diseases and even in those of healthy individuals. These investigators did not consider these organisms of any pathological significance and interpreted them as stages in the development of flagellates.

Aside from the observations mentioned, the first investigator to describe minutely amebae found in the human intestine, and to associate them with the etiology of dysentery, was Loesch,<sup>7</sup> in 1875, who studied carefully the amebae found by him in the stools of a man suffering from chronic diarrhea. To these organisms he gave the name "*Amoeba coli*," and claimed to have been able to produce dysentery in dogs by the rectal injection of fecal material containing them. Grassi<sup>8</sup> confirmed the presence of amebae in human dejecta, but considered them as harmless, as he found them in both health and disease. Grassi's paper was followed by those of Sonsino, Norman,<sup>9</sup> Perroncito,<sup>10</sup> Callandrucio,<sup>11</sup> and Blanchard, all confirming the presence of amebae in the stools of patients suffering from diarrhea or dysentery.

In 1883, Robert Koch,<sup>12</sup> while investigating cholera in Egypt, found amebae in the tissues of the intestine in three cases of dysentery, and was able to demonstrate them in sections, situated at the base of the ulcerations, which situation Koch considered as conclusive of their etiological relationship to the disease. Koch's paper resulted in the publication by Kartulis<sup>13</sup> in 1886, of his investigation of Egyptian dysentery, in which he states that in all of 150 cases of dysentery he was able to demonstrate amebae in the stools; he concludes that the amebae are the cause of the dysentery and in later publications<sup>14</sup> gave the results obtained in the study of 500 cases of the

disease. The work of Kartulis may be said to have finally established the etiological relationship of amebae to dysentery and his results were soon confirmed by numerous investigators in various parts of the world.

Hlava<sup>15</sup> found amebae in 60 cases of dysentery occurring in Prague, and produced dysentery in both cats and dogs by the rectal injection of feces containing amebae; Osler<sup>16</sup> in 1890, was the first to find the parasite in America, observing amebae in the feces, and in the pus from a liver abscess, in a man suffering with dysentery. Other American observers, as Musser,<sup>17</sup> Stengel,<sup>18</sup> and Dock<sup>19</sup> confirmed the presence of these organisms in dysenteric stools. In 1891 appeared Councilman and Lafleur's<sup>20</sup> classical work upon amebic dysentery, in which the authors conclude that the disease is a clinical entity, and is characterized by a definite pathology, the lesions of which are due to amebae. They proposed the name "*Amoeba dysenteriae*" for the ameba associated with the lesions they described, and stated that other, and perhaps non-pathogenic amebae might infest the intestine of man. Confirmatory studies appeared from 1891 to 1893 by Cahen,<sup>21</sup> Lutz,<sup>22</sup> Kovacs,<sup>23</sup> and Quincke and Roos,<sup>24</sup> and in 1894, Kruse and Pasquale<sup>25</sup> published the results of their investigations of dysentery in Egypt, in which they also conclude that amebic dysentery is due to a specific ameba, and that other amebae occur in man which are harmless. The later investigations of Harris<sup>26</sup> and the writer,<sup>27</sup> in the United States, of Strong and Musgrave<sup>28</sup> and Musgrave and Clegg,<sup>29</sup> in the Philippines, have all been confirmatory of the etiological relationship of amebae to a characteristic form of dysentery, especially prevalent in tropical regions, and frequently accompanied by abscess of the liver.

#### CLASSIFICATION AND NOMENCLATURE.

Almost from the time of the first description of the amebae in man the classification and nomenclature of these organisms has occasioned much confusion and difficulty. While all were agreed that they belonged to the Protozoa, almost every writer differed in his classification or in his conception of the biological history of the amebae associated with dysentery, and it was not until Schaudinn's observations were published that a clear and definite classification of these parasites was possible.

The absence of amebae in numerous well-marked cases of dysentery, and their presence in health and in diseases other than dysentery, had gradually led to the grouping of students of the subject into three schools, i. e., those believing that amebae are always harmless commensals, more numerous, perhaps, in the feces of dysenteric patients because of a more favorable environment; those believing that all amebae may be pathogenic if suitable conditions be present; and those believing that harmless and pathogenic species are present in the intestine of man.

Prior to Schaudinn's work several observers had endeavored to

establish a classification based upon morphological differences, but without success so far as the general acceptance of any one classification was concerned. The following is a brief résumé of the attempts made in this direction:

In 1893, Quincke and Roos,<sup>24</sup> as the result of their studies, divided the amebae infecting man into three species, as follows:

1. *Amoeba intestini vulgaris*, 40 microns in diameter, with large granules, which is pathogenic for neither man nor cats.

2. *Amoeba coli mitis*, similar in size and appearance to the preceding, but which is pathogenic for man alone.

3. *Amoeba coli* (Loesch) about 25 microns in diameter, with a finely granular endoplasm, which produces dysentery in both man and cats.

This classification, which was based largely upon the results of animal experiments, is not conclusive, as they evidently, from their descriptions of the organisms, were dealing with mixed infections with both the harmless and pathogenic ameba.

Celli and Fiocca<sup>30</sup> described no less than six species of amebae infecting the intestine of man. Their classification is as follows: (1) *Amoeba spinosa*; (2) *Amoeba vermicularis*; (3) *Amoeba diaphana*; (4) *Amoeba reticularis*; (5) *Amoeba lobosa*, variety *guttata*; (6) *Amoeba lobosa*, variety *oblonga*. The names sufficiently describe their differential characteristics, and they all differed in size, one species, *A. diaphana*, measuring only 0.5 to 2 microns in diameter. To one who has studied these organisms the description of an ameba measuring but 0.5 micron in diameter may well be viewed with suspicion, when we consider that such an organism would be but little larger than the *Micrococcus melitensis*, which measures 0.4 micron in diameter. The largest ameba described by Celli and Fiocca, *A. spinosa*, did not exceed 10 microns in diameter, which is positive proof that these authors were not dealing with true dysentery amebae and it is more than probable that many of the amebae described by them were really stages in the development of the intestinal flagellates.

In 1894, Kruse and Pasquale<sup>31</sup> distinguished four varieties of amebae based entirely upon morphological characteristics: (1) A form presenting a very refractive protoplasm, found in normal feces; (2) A form showing irregular and small granules; (3) A form in which the endoplasm consisted largely of vacuoles; (4) A form in which the protoplasm was filled with foreign bodies. The two latter forms were found only in dysenteric feces.

It will at once be seen that the differences in these forms are so slight as to be of no scientific value as a basis of classification, and the fact that these authors describe the form found in normal feces as being very refractive is evidence that they confused the pathogenic and harmless species, although they recognized a pathogenic ameba, *A. dysenteriae*, thus following Councilman and Laflaur<sup>20</sup> who, in 1891, objected to *Amoeba coli* as a name for the ameba causing dysentery and suggested the name "*Amoeba dysenteriae*." The latter authors, while not attempting to differentiate species, expressed it as their opinion that under certain conditions a number of species of amebae may inhabit the intestine.

Casagrandi and Barbagallo were the first investigators to describe accurately the species of ameba occurring in the feces of healthy individuals, and in those of patients suffering from other diseases than dysentery, although Grassi<sup>8</sup> first called attention to

such amebae. They established for this ameba the genus *Entamoeba* which was afterward accepted by Schaudinn. They describe very minutely the morphology of this species and also the method of reproduction, which they state consists normally in simple division and in the formation of cysts in which eight daughter amebae develop. Their investigations directed the attention of other workers to the species of amebae occurring in man, but they were not successful in differentiating a pathogenic and harmless species. Strong and Musgrave<sup>33</sup> recognized two species of amebae as occurring in their cases in Manila and state that with the harmless ameba, which they call *Amoeba coli*, found in normal individuals, they were never able to produce dysentery in cats, while with the pathogenic ameba, *Amoeba dysenteriae*, "we have had no difficulty in producing dysentery and ulceration of the large bowel in cats by injection of the stools or contents of liver abscesses containing motile *Amoebae dysenteriae*."

Strong,<sup>34</sup> in a later publication, divides the amebae in man into two species, *Amoeba dysenteriae* and *Amoeba coli*, the first causing dysentery; the second, non-pathogenic. He describes in detail the morphology of these organisms and gives the results of animal experiments.

According to Schaudinn<sup>1</sup> the first investigator to clearly identify and describe the two species of amebae infecting man was Jürgens<sup>35</sup> and much of Schaudinn's work is confirmatory of that of the latter observer. To Schaudinn, however, undoubtedly belongs the credit of the establishment, on scientific grounds, of the two distinct species of amebae which infect the intestine of man.

In describing in detail the morphology of the amebae considered in this paper I shall speak more fully of Schaudinn's results, which lead him to classify the amebae in man into two species under the genus *Entamoeba* established by Casagrandi and Barbagallo. To the amebae occurring in the feces of normal individuals, or in those suffering from other diseases than dysentery, he gave the name *Entamoeba coli*, while to those occurring in dysenteric feces, and causing that disease, he gave the name *Entamoeba histolytica*. Regarding the nomenclature of the amebae in the human intestine Stiles<sup>3</sup> has written an exhaustive article in which he clearly states the nomenclatural situation, and concludes that for those who believe that there is but one species of ameba infecting man the correct name to use is *Entamoeba coli*, not *Amoeba coli*, for, as shown by Casagrandi and Barbagallo,<sup>32</sup> the amebae of the human intestine differ from the freshwater amebae to which the generic terms "Chaos," later emended by Ehrenberg to "Amoeba" were originally given; while for those who believe in a harmless and pathogenic species, the correct generic terms are *Entamoeba coli* and *Entamoeba histolytica* respectively. In a previous contribution<sup>36</sup> I suggested that as the name *Amoeba dysenteriae* had been given by Councilman and Lafleur, the name *histo-*

*lytica* should give way to *dysenteriae*, but as Stiles has conclusively shown that *dysenteriae* is not a *new* name but merely a *synonym* of *Amoeba coli*, it follows that it cannot be used to designate the pathogenic ameba, and therefore we must accept Schaudinn's name *Entamoeba histolytica* for the parasite causing amebic dysentery.

At the present time Schaudinn's classification has been accepted by most investigators and writers, and by all zoölogists whose writings upon the subject I have seen. Musgrave and Clegg,<sup>29</sup> almost alone of those who have had an extensive experience with amebic dysentery, still refuse to accept Schaudinn's classification, although they state, "we do not at all question the multiplicity of both genera and species of amebae, both within and without the intestine of men." They also adhere to the name *Amoeba coli* although, as Stiles has shown, the proper generic term is *Entamoeba*.

The exact zoölogical position of the intestinal amebae of man may be indicated as follows.

#### THE PROTOZOA.

Class. *Sarcodina*.

Sub-class. *Rhizopoda*.

Order. *Amoebida*.

Family. *Amoebidae*.

Genus, *Entamoeba* (Casagrandi and Barbagallo, 1897).

Species,

*Entamoeba coli* (Schaudinn, 1902).

*Entamoeba histolytica* (Schaudinn, 1903).

#### OCCURRENCE OF AMEBAE IN THE HEALTHY INTESTINE.

Before the publication of Schaudinn's paper a few observers had noted the occurrence of amebae in the intestinal discharges of healthy individuals and this fact gave rise to the opinion that amebae were harmless commensals and only of accidental occurrence in patients suffering from dysentery. However, as evidence of the pathogenic action of amebae accumulated it was found that such an opinion was untenable. In order to explain pathogenesis the theory regarding the effect of environment upon these organisms arose, together with the belief that more than one species of amebae occurred in the human intestine.

Comparatively little work has been done regarding the frequency of the occurrence of amebae in the feces of healthy individuals, and it is remarkable that even Schaudinn's paper seems to have stimulated but little research in this direction. The examination of the feces in health results almost invariably negatively as regards the presence of amebae, unless some drug be given which will produce a slight diarrhea. This undoubtedly accounts for the negative results obtained by some careful observers, for I have examined a large number of healthy men in which no amebae were found unless a cathartic was first administered. Such experiments show that in order to demonstrate *E. coli* in the feces in health it is necessary that a condition of diarrhea be induced by the use, preferably, of a saline cathartic. Negative results obtained by observers who have not followed this procedure are of no value.

*Historical summary.*—Grassi,<sup>8</sup> in 1888, was probably the first investigator to demonstrate the occurrence of amebae in the feces of healthy individuals, and he gave a very full and accurate description of the organism now known as *Entamoeba coli*, although Cunningham,<sup>37</sup> in 1881, stated that he found amebae in the stools of healthy men, but his descriptions indicate that he was probably observing developmental stages of flagellates. Schuberg<sup>38</sup> demonstrated amebae in the feces of ten out of twenty healthy individuals examined, or 50 per cent, while Gasser<sup>39</sup> examined the feces of twenty healthy persons and found amebae in 20 per cent of them. Strong and Musgrave<sup>33</sup> found them in only 4 per cent of healthy persons examined, yet felt justified in believing that these amebae were harmless because of the negative results of animal experiments. Dock<sup>40</sup> examined the stools of 200 healthy individuals and was only able to find amebae in two; he concludes his contribution as follows: "Even if a certain parasite occurs in every case in one locality, it would not follow that the same parasite would also be found as wide-spread elsewhere." Kartalis<sup>43</sup> examined the stools of several hundred healthy individuals and was able to demonstrate amebae in only three cases, but he neglected to produce a diarrheal condition before the examinations, which undoubtedly accounts for the small positive result. Schaudinn<sup>1</sup> in 1903, published the results obtained by him in the examination of the feces of healthy individuals; he found, at his home in West Prussia, that 50 per cent of the healthy individuals examined among the farming population showed the presence of *E. coli* in their feces, while in Berlin he found that only 20 per cent showed this parasite. Along the shores of the Adriatic he found that in 385 examinations in as many individuals in perfect health, no less than 256, or 66 + per cent, showed the presence of *E. coli*. His observations show that locality and occupation had something to do with the number of individuals infected.

*Personal observations.*—In 1905, I published<sup>2</sup> the results obtained in the examination of the feces of American soldiers stationed at San Francisco, Cal., which were confirmatory of those obtained by Schau-



dinn in Europe. My experiments were conducted largely upon members of the Hospital Corps of the U. S. Army, recruited from almost every portion of the United States, who were on duty at the General Hospital at that time, and were under constant observation. I made over 200 examinations in such men, and found that, after the administration of magnesium sulphate in ounce doses, the feces of 65 per cent showed the presence of *E. coli*, but in many cases repeated examinations were necessary in order to demonstrate the parasite. Thinking that geographic distribution might have something to do with the proportion of infected individuals, I inquired into the locality from which the individuals came, but was unable to prove that the proportion of infected cases varied to any marked extent with the locality. This negative result cannot be considered as conclusive, and the question can only be settled definitely when large numbers of persons can be examined, under similar conditions, in different localities. All of the men I examined were in robust health, gave no history of diarrhea or dysentery, and certainly presented no symptoms of either condition. Many of the men showed immense numbers of *E. coli* in the feces, while in others they were very few in number.

An examination of my own feces, and of that of other medical men on duty at the hospital, resulted in the demonstration of this ameba and at the date of this writing, 1908, none of us have developed dysentery. My results, as showing the proportion of American soldiers harboring this parasite, were confirmed by Vedder,<sup>41</sup> who, in 1906, published the results obtained by him in the Philippine Islands in the examination of American and native soldiers. After giving them preliminary doses of magnesium sulphate he examined the feces of 50 healthy American soldiers, and 50 Filipino scouts; of the American soldiers, 50 per cent showed *E. coli* in their feces, while of the Filipino scouts, 75 per cent were infected with this parasite. Regarding the subsequent history of these men Vedder says: "all the men have been under observation for a period of nine months and none of them has developed dysentery." Ashburn and myself, while serving in Manila upon the "Army Board for the Study of Tropical Diseases," examined the feces of 107 healthy American soldiers. The result of our work, published in 1907,<sup>42</sup> was briefly as follows:

In all we examined 107 healthy men, all members of the Hospital Corps of the Army, and all on active duty at the U. S. Army Division Hospital, Manila, P. I. Of the 107 men, 76 or 71 + per cent were found to be infected with the non-pathogenic *Entamoeba coli*, while two showed the pathogenic *Entamoeba histolytica* in their stools. None of these men, with the exception of the two showing the pathogenic ameba, had diarrhea or dysentery at the time of examination, and all denied ever having suffered from dysenteric symptoms since residing in the Philippines. Of seventy-two men showing *Entamoeba coli* in their feces, one had resided in the Philippine Islands for eight years; four, seven years; one, six and a half years; three, six years; four, five and a half years; one, five and one quarter years; two, five years; four, four years; three, three years; two, two and a half years; ten, two years; one, one year and ten months; two, one year and nine months, nine, one and a half years; thirteen, one year; and the remainder, or seventeen, less than one year.

The two men showing *Entamoeba histolytica* in their stools were apparently in good health but inquiry elicited the information that both were suffering from dysenteric symptoms at the time of examination, and both were later returned to the United States suffering from chronic amebic dysentery. At the time that we examined the feces of these men we knew nothing of the occurrence of the dysenteric symptoms, and our diagnosis was based entirely upon the morphology of the amebae observed in their feces. It will thus be seen, that contrary to the opinion of certain investigators, it is possible to differentiate *Entamoeba coli* from the *Entamoeba histolytica* as they occur in the feces, and therefore, that such differentiation becomes of very great importance in the diagnosis of diarrheal conditions of the intestine.

In order to determine how long infection with *Entamoeba coli* might exist we made the following examinations:

A. Upon Nov. 20, 1906, thirteen men were re-examined who had been first examined upon March 17, 1906, eight months having elapsed since the first examination. Of these thirteen men, eleven showed *Entamoeba coli* in their feces March 17th, and nine, or 81.8 per cent still showed them upon Nov. 20th. During this time not one of these men had suffered from diarrhea and all had been on duty continuously at the hospital.

B. Upon Nov. 20, 1906, seven men were re-examined who were first examined upon May 2, 1906, six months and twenty-two days having elapsed since the first examination. Of these seven men, five were positive for *Entamoeba coli* upon May 2nd, and five were still positive upon Nov. 20th, and none of these men had suffered from diarrhea or dysentery during this time, and were continuously under observation.

C. Upon Nov. 20, 1906, eight men were re-examined who were first examined upon July 10, 1906, four months and thirteen days having elapsed since the first examination. Of these eight men, five were positive for *Entamoeba coli* upon July 10th, and two were still positive upon Nov. 20th. Neither of these men had developed symptoms of diarrhea or dysentery during this time.

As the result of our work in the examination of the feces of healthy men in the Philippine Islands, Ashburn and I concluded that a very large proportion of white men in these islands harbor *E. coli*, which condition, so far as we were able to observe, does not result in symptoms of diarrhea or dysentery, for in many of the cases the amebae

disappeared without producing symptoms, while in the greater proportion of the cases they persisted for as long as nine months, as shown by us, during which time no disease of the intestine resulted. We also concluded that *E. coli* and *E. histolytica* differ greatly as regards morphology, and that it is possible to distinguish these two species of amebae by their morphological characteristics as observed in fresh specimens of feces, and that "latent infection" cannot explain the lack of pathogenicity in persons harboring *E. coli*.

Summing up my observations of 1905, in San Francisco, and those undertaken with Ashburn in Manila, we have the record of 307 examinations of feces from as many healthy men, all American soldiers, with the result that 176 or 58+ per cent showed the presence of the harmless *E. coli* in their feces. These amebae invariably answered to the description of *E. coli* as given by Schaudinn and myself, and the great majority of the men in whom they were found were under observation for from six months to a year or more, during which time no symptoms of dysentery developed. In 1904 an examination of my own feces after the administration of magnesium sulphate resulted, as has been noted, in the demonstration of *E. coli* in them, and repeated examinations since then, a period of nearly four years, has always resulted in finding numerous typical *E. coli*, and not the slightest symptoms of dysentery have developed.

It would appear to me that these observations prove that amebae may be present and multiply in the intestine of man without producing symptoms, for periods longer than the known incubation period of amebic dysentery, for to hold that the incubation period of this disease may extend over a period of years is impossible, especially when we consider the chances of infection in the meantime with the pathogenic species.

Musgrave and Clegg,<sup>43</sup> in attempting to disprove the existence of a harmless ameba in man, instance cases in which amebae were found in the feces during apparent health but in which dysentery followed at periods varying from two to six months, but such cases are of no scientific value as proof of their contention for during the entire period which they regard as the period of incubation the patients were continually exposed to infection with *E. histolytica* in an intensely infected region, i. e., the Philippine Islands. In a later contribution<sup>44</sup>

Musgrave and Clegg attempt to refute the observations of Schaudinn and myself as regards the occurrence of amebae in healthy individuals by the results obtained in the examination of inmates of the hospitals and prisons of Manila. They admit that the examinations "undertaken to determine the prevalence of amebae in the intestine have not dealt with the question of whether or not this situation is a normal one." Their results were briefly as follows:

Of 587 cases in Bilibid Prison, 26+ per cent showed amebae, but these examinations are not said to have been upon healthy men. In another series of 100 cases, 39 per cent showed amebae. Of 318 American patients in the Civil Hospital, most of them suffering from intestinal disturbances, amebae were found in 14+ per cent; of 143 stools examined at St. Paul's Hospital, 44 per cent were positive, and of 30 examinations made at the Biological Laboratory "from stools sent in by private patients suffering from diarrhea or dysentery, 7 were positive or 23+ per cent."

From the data given it will be seen that these observers have compared with the results of Schaudinn and myself, in the examination of the stools of healthy individuals, their results in the examination of the stools of patients, the majority of whom they admit were suffering from diarrhea or dysentery. In a critical paper Vedder<sup>58</sup> has analyzed these results and I can do no better than quote him in showing that no scientific deductions can be safely drawn from such a comparison. He says:

The examinations of amebae detailed by the authors cannot possibly afford any conclusions as to the presence or absence of amebae in healthy individuals. They examined 587 cases from Bilibid Prison, but they say nothing as to how many cases were normal or how many suffered from disease, or were under treatment for diarrhea or dysentery. If these individuals were normal, the observations of Craig and Vedder are thereby confirmed. If they were the subjects of dysentery, by what possible logic can it be inferred that amebae could not be found in healthy individuals.

I believe that it is evident, from the observation given, that in a certain proportion of healthy individuals, in certain localities, an examination of the feces will demonstrate that the intestine harbors a harmless ameba, and that this organism answers to the description given by Schaudinn for *E. coli*. Schaudinn found this ameba present in 50 per cent of healthy individuals examined in West Prussia, in 20 per cent examined in Berlin, and in 66 per cent of those examined along the shores of the Adriatic; Craig in 65 per cent of healthy American soldiers at San Francisco; Ashburn and Craig in 71 per cent of healthy American soldiers in Manila. P. I.; and Vedder in 50 per cent of

American soldiers, and in 72 per cent of Filipino scouts in Mindanao, P. I. While it is obvious that the percentage of infections varies in different localities, probably depending on local conditions and the presence of a source of infection, the fact remains that infection with *E. coli* is very common and that it is not difficult to demonstrate this organism in the feces of healthy individuals after the administration of a saline cathartic.

#### OCCURRENCE OF AMEBAE IN DISEASES OTHER THAN DYSENTERY.

As regards the occurrence of *E. coli* in the feces of patients suffering from other diseases than dysentery, several observers agree in having demonstrated amebae in diseases such as chronic enteritis and typhoid fever, and also in conditions in which the lesions were not confined to the intestine.

Cunningham<sup>37</sup> found amebae in the intestinal contents of cholera cases; Celli and Fiocca<sup>39</sup> in many infants suffering from intestinal inflammation; Berndt<sup>45</sup> in cases of typhoid fever; Normand<sup>9</sup> in chronic colitis; Massiutin,<sup>46</sup> working in Kiev, in the feces of five cases suffering from intestinal catarrh, typhoid fever, and diarrhea; Grassi<sup>8</sup> in diarrhea; Peroncito<sup>10</sup> in chronic diarrhea; Babes in cases of hepatitis, Cassagrandi and Barbagallo<sup>32</sup> in various diseases, as well as Kruse and Pasquale,<sup>31</sup> Quincke and Roos<sup>24</sup> Kartulis,<sup>13</sup> Shuberg,<sup>38</sup> Gros,<sup>47</sup> Sternberg,<sup>48</sup> and Ijima.<sup>49</sup>

In my investigations I have not confined myself to examinations of the feces of patients suffering from diarrhea, but have examined the excreta in all cases, whatever the diagnosis, and I have found that of 250 such cases, 49 per cent showed *E. coli*. It is significant that a smaller percentage of cases of disease show this ameba than of healthy individuals, which would appear to indicate that *E. coli* finds a more congenial environment for its development in the intestine of normal individuals. Table I, giving the results obtained in the examination of 30 cases, well illustrates the variations in disease from which the patients suffered, and also the different localities from which the patients came. In all the cases where diarrhea was not present a saline cathartic was given before the examination. From a consideration of this table it is evident that none of these cases were suffering from diarrhea or dysentery, and that they were under observation in the hospital for periods varying from 10 days to 5 months, during which time no dysenteric symptoms developed; it cannot then be said that these patients were cases of latent infection or that the symptoms indicated an inflamed intestinal tract; indeed I have found that

*E. coli* occurs fully as frequently in the feces of patients suffering from other diseases not diarrheal in character as it does in those cases presenting diarrhea provided a saline cathartic be given before the examination. The occurrence of such a large number of instances of the presence of *E. coli* in patients suffering from other diseases is of great practical importance, especially in those localities where amebic dysentery is endemic or in patients coming from such regions. An examination of the feces in this class of cases, if diarrhea were present, would

TABLE I.  
EXAMINATION OF PATIENTS FOR *E. Coli*.

No.	Diagnosis	Residence	Dysentery	Diarrhea	In Hospital	Bowel Movements	<i>E. Coli</i>
1.....	Anemia, secondary	England	No	No	70 days	2 daily	Present
2.....	Abscess of leg	Illinois	"	"	5 months	2 "	"
3.....	Abscess of axilla	.....	"	"	20 days	2 "	"
4.....	Diabetes insipidus	Georgia	"	"	40 days	1 "	"
5.....	Fracture	Alabama	"	"	40 days	2 "	"
6.....	Gastritis, chronic	.....	"	"	2 months	2 "	"
7.....	"	.....	"	"	2 weeks	1 "	"
8.....	Gastritis, acute	California	"	"	1 week	1 "	"
9.....	Gonorrhea, acute	Tennessee	"	"	1 month	1 "	"
10.....	"	California	"	"	1 month	1 "	"
11.....	"	Jamaica	"	"	10 days	2 "	"
12.....	"	Pennsylvania	"	"	20 days	1 "	"
13.....	" Chronic	Kentucky	"	"	35 days	2 "	"
14.....	"	.....	"	"	1 month	1 "	"
15.....	"	Pennsylvania	"	"	1 month	1 "	"
16.....	Hemiplegia	California	"	"	10 days	1 "	"
17.....	Pharyngitis, acute	.....	"	"	10 days	1 "	"
18.....	Malarial Fever	Connecticut	"	"	2 weeks	1 "	"
19.....	"	California	"	"	2 weeks	2 "	"
20.....	"	Virginia	"	"	2 weeks	1 "	"
21.....	"	.....	"	"	2 weeks	1 "	"
22.....	Otitis media	Texas	"	"	3 months	1 "	"
23.....	Measles	"	"	"	40 days	1 "	"
24.....	"	"	"	"	4 months	2 "	"
25.....	Pemphigus	Arkansas	"	"	5 months	1 "	"
26.....	Poliomyelitis	Mississippi	"	"	4 months	2 "	"
27.....	Mitral regurgitation	Pennsylvania	"	"	10 days	2 "	"
28.....	Stricture	California	"	"	2 weeks	1 "	"
29.....	Sciatica, chronic	Missouri	"	"	3 months	1 "	"
30.....	Varicocele	Ohio	"	"	3 months	2 "	"

result in the finding of amebae which the inexperienced observer would probably regard as *E. histolytica*, and a diagnosis of amebic dysentery would be made. Since I have been investigating this subject I have seen numerous cases diagnosed as amebic dysentery, because of the finding of *E. coli* in the stools after a dose of salts, which were in reality some other disease, and the clinical history of these cases was conclusive evidence that the diagnosis of amebic dysentery was erroneous. I am convinced that a considerable proportion of the cases returned from the Philippine Islands, during 1900 and 1901, and perhaps later, diagnosed as amebic dysentery were, in reality,

cases of enteritis showing *E. coli* in the feces, and the clinical histories bear out this belief. It is certainly necessary, then, that we recognize the fact that non-pathogenic amebae do occur both in health and disease in the intestine of man, and that we should be able to differentiate between *E. coli* and *E. histolytica*; for this reason I have endeavored in the following descriptions of these organisms to indicate the principal differential features but it will be found that considerable experience will be required in the examination of the feces both in health and in amebic dysentery before such a differentiation will become easy, and it should be remembered that no merely superficial study of these organisms will result in success in this direction.

#### METHODS OF EXAMINATION.

The amebae infesting the intestine of man may be studied in both fresh and stained specimens of the feces; for the study of the vital activities of these organisms the fresh specimens are best, while for the study of the exact structure and the methods of reproduction the stained specimens are necessary. With either method a differential diagnosis of *E. coli* and *E. histolytica* can be made.

*Fresh preparations.*—A very small portion of a freshly passed stool should be placed upon a microscopic slide and covered with a cover-glass, gentle pressure being used to spread the specimen. In making the preparation take preferably a drop of the liquid portion of the stool, and if this is impossible, select a fragment of mucus or of any blood-stained material. The preparations should be examined with a one-sixth-inch lens and a one- or two-inch eyepiece. For the finer details regarding the structure of the protoplasm and the nucleus it is necessary to use the one-twelfth-inch objective. The use of a very weak solution of neutral red is often of great service in those cases in which the amebae are few in number; this solution is very quickly absorbed by the amebae, coloring them a fine pink, and if it is not too strong it will not interfere with the movements of the organisms, so that it is very easy to distinguish them, as the only other organisms that take up the stain are the flagellates and these are easy to differentiate from either *E. coli* or *E. histolytica*. Even when the amebae are motionless it is not difficult to distinguish them by the use of this reagent. If the feces have to be kept for any time the vessel containing

them should be placed in another containing water at a temperature of at least 100° F., which temperature should be maintained. However, it is often possible to render the amebae motile, even in feces kept at room temperature for hours, by gently warming the slide before placing it upon the microscope. Many observers claim that a diagnosis of the presence of amebae in feces should never be made unless the organisms be motile. While this is valuable advice for the novice in this line of work, it is certainly true that by one who has studied these organisms, they can be recognized easily when motionless, provided that the feces have not been kept so long as to have lead to degenerative changes in the amebae.

*Staining methods.*—So far as gross staining is concerned, both *E. coli* and *E. histolytica* may be stained by solutions of many of the anillin dyes, as methylene blue, Borrel blue, carbol-fuchsin, thionin, etc., but the only stain that I have found satisfactory in demonstrating the exact structure of these organisms and their method of reproduction, is Wright's modification of the Romanowsky stain, made as directed by Oliver, of San Francisco. With this stain the chromatin of the nucleus is differentiated in some specimens very beautifully, as well as the granular structure of the protoplasm. Both species of amebae stain with some difficulty with this stain, and sometimes scores of specimens will have to be examined before a perfectly stained one is found; for this reason the use of stained preparations in the differentiation of these organisms is not to be recommended for general work, as it requires time and detail of technic not possessed by the inexperienced observer. Löffler's solution of methylene blue, carbol-fuchsin, Borrel blue, and thionin are good general stains but they are really of little diagnostic value. The thionin method, recommended by Mallory and Wright,<sup>50</sup> and Heidenhain's iron hematoxylin and the Borrel stain, as recommended by Woolley and Musgrave<sup>51</sup> give the best results in the staining of amebae in the tissues.

*Method of preparing spreads for staining.*—Several cover-glasses are thoroughly cleaned and placed in a row upon the table. With a sterilized platinum loop a small portion of the material to be examined is picked out of the feces and placed upon the cover-glass. A clean cover-glass is placed over this, and the two slid carefully apart, using gentle pressure, and allowed to dry. By proceeding in this way a dozen or more even, thin smears can be made in a very few minutes. If any other method than Wright's is used the preparations should be placed smeared side up in equal parts of



alcohol and ether and allowed to remain for from 15 to 20 minutes in order to fix the specimen. If Wright's method be used it is not necessary to thus fix the specimen, if the feces are warm many of the amebae will be fixed while undergoing ameboid motion. After fixing, the smears are ready to stain.

As I shall only describe the staining reactions of *E. coli* and *E. histolytica* with Oliver's modification of Wright's method it is unnecessary for me to give in detail the various steps in the application of other methods. The stain I have used is prepared as follows:

Add 0.5 gm. of sodium bicarbonate to 100 c.c. of distilled water, dissolve thoroughly, and add 1 gm. of methylene blue (Grubler's); heat the mixture for one hour in an Arnold sterilizer after the steam is up. After heating allow the solution to cool. Make a 1 to 1,000 solution of yellow aqueous eosin (Grubler's) and add this, while stirring, to the cooled methylene blue solution in the proportion of 500 c.c. of the eosin solution to 75 c.c. of the blue solution. This should be done in a large white porcelain dish and the eosin solution added until a marked metallic scum appears upon the surface, even though more of the eosin solution has to be added than is recommended. After letting the mixture stand for a period of from 10 to 20 minutes, filter it through one small filter paper, save the precipitate on the paper, dry in a hot air oven, and use to make the staining solution. This powder will keep well and can be used as stock material.

To prepare and use the staining solution, proceed as follows:

Take 0.3 gm. of the powder and add it to 100 c.c. of pure methylic alcohol (Mercks reagent alcohol is best, although any methylic alcohol absolutely free from acetone will do) filter, and to 80 c.c. of the filtrate add 20 c.c. of methylic alcohol, or enough to bring the amount up to 100 c.c. The staining solution is now ready for use and will keep unimpaired for several weeks.

To stain,

add a few drops of the staining solution to the preparation of feces without preliminary fixation, and let stand for five minutes; then add enough distilled water to cause a slight metallic scum to form on the surface of the preparation; let stain for 10 minutes and wash thoroughly in running distilled water. If it is desired to preserve the specimens do not use a cover-glass for making the smear but a slide, and do not mount in Canada balsam, with a cover-glass, but preserve the slide as it is.

For the diagnosis of amebae in the stools, staining methods should never be resorted to, as the examination of the fresh specimen is altogether superior and safer. Stained preparations are only of use in the study of certain morphological characters and the methods of reproduction.

#### DESCRIPTION OF ENTAMOEBA COLI.

*General description.*—*E. coli* consists of a mass of protoplasm, containing a well-defined nucleus, and in the majority of instances, one or more nucleoli. A vacuole, non-contractile in character, is

present in some instances, but multiple vacuoles are seldom, if ever, observed. The protoplasm of the organism is divided into two portions, an outer, or ectoplasm, and an inner, or endoplasm, but this differentiation cannot be made out, in most instances, even when the organism is in motion. While possessing the power of motion *E. coli* is very sluggish as compared to *E. histolytica*, and, as a rule, may be said to be almost immotile. Reproduction occurs, under favorable conditions, by simple division, while if unfavorable conditions arise, reproduction occurs by encystation, with the formation within the cyst of eight daughter-cells, which are liberated and become young amebae. The following details in the morphology of this organism have been determined from the study of thousand of specimens, but it should be remembered that not every ameba of this species presents all the details described, but that the description is a composite picture, true for the vast majority of organisms examined.

*Size.*—*E. coli* is never as large as the largest specimens of *E. histolytica*, but while this is so, it is not at all uncommon to find *E. coli* that approximate the size of the majority of *Entamoeba histolytica* that are usually observed. Almost every writer upon the amebae of man have differed in the measurements given for these organisms as is well shown by the following summary:

Kartulis<sup>13</sup> gives the size of the ameba as from 12 to 30 microns in diameter; Quincke and Roos,<sup>24</sup> from 15 to 25 microns; Celli and Fiocca<sup>30</sup> 0.5 to 10 microns; Councilman and Lafleur,<sup>20</sup> 6 to 35 microns; Kruse and Pasquale,<sup>31</sup> from 10 to 50 microns; Osler,<sup>16</sup> from 10 to 20 microns; Sodre,<sup>52</sup> from 15 to 35 microns; Marshall<sup>53</sup> from 10 to 40 microns; Fitcher,<sup>54</sup> from 12 to 26 microns; Zorn,<sup>55</sup> 14 to 22 microns; Strong and Musgrave,<sup>28</sup> 10 to 50 microns, and Musgrave and Clegg,<sup>29</sup> 3 to 40 microns.

The measurements given do not indicate inaccuracy upon the part of the observer but rather that amebae of various size occur in almost every specimen of feces examined. Schaudinn<sup>1</sup> gave the size of *E. coli* as varying between 8 and 50 microns, but it is very seldom that one of these organisms is observed exceeding 25 to 30 microns in diameter, and as a rule this organism measures from 10 to 20 microns in diameter. This average is smaller than for *E. histolytica*, the majority of which measure from 25 to 35 microns, and is, therefore, of some diagnostic importance in distinguishing between them, but the mere difference in size cannot be depended upon in such differentiation, for as I have stated, *E. coli* approximates *E. histolytica* in size.

Therefore, as I have before contended, the separation into species of the amebae of the human intestine, based alone upon size, is altogether unscientific and erroneous. When encysted, *E. coli* measures on the average from 10 to 15 microns, and is always smaller than before encystment.

Those authors who claim that the pathogenic amebae is always larger than the non-pathogenic are surely in error for I have repeatedly seen small *E. histolytica* predominating in feces from well-marked cases of amebic dysentery, and an examination of the feces of this disease will show that amebae of large size are by no means always present, and that generally both large and small amebae are present. It follows, then, that the size of an ameba is of little value in specific diagnosis, although the vast majority of *E. coli* average smaller than *E. histolytica*.

The variation in size of these organisms is, of course, explained by the fact that various stages of development occur simultaneously in the stools; the fact that the greater number approximate each other in size indicates that reproduction of the parasite occurs at definite periods of time and is followed by uniform growth. As yet we have no data which justifies a classification of amebae based upon the measurements of the organisms, and the most that we can say is: that *E. coli* is generally considerably smaller than *E. histolytica*.

*Shape*.—When not in motion *E. coli* is invariably perfectly spherical in shape. When in motion the shape is very variable, depending upon the contour of the extruded pseudopodia, which are always rounded, and never spinose, as are the pseudopodia of so many of the amebae cultivated from external sources and used, by some authors, in comparison with this parasite. *I have never seen an ameba in the feces with spinose pseudopodia, and yet, in cultures, this is the prevailing type, thus indicating that if these cultivated amebae are really E. coli or histolytica they undergo very marked changes in morphology during cultivation.*

*Color*.—*E. coli* is always of a peculiar dull grayish color and this is true of the organism whether the feces contains blood or whether the intestine is normal. The color of this organism is of value in distinguishing it from *E. histolytica*, especially in those cases of dysentery in which these two varieties may occur together. Musgrave and

Clegg<sup>50</sup> considers that color simply means environment, the amebae becoming greenish in color as the stool becomes bloody, due to absorption of blood serum containing hemoglobin, but I have yet to see an ameba answering to the description of *E. coli* of a greenish color, and experimentally I have demonstrated that the addition of blood to feces containing this organism does not result in the absorption of either dissolved hemoglobin or red blood corpuscles. It is extremely difficult to describe the exact color of this organism but to one who has studied both species the difference in the coloring of *E. coli* is very characteristic. Irrespective of the greenish coloration of *E. histolytica*, so often observed, there is a marked difference in the color of these two organisms.

*Protoplasm*.—As pointed out by Schaudinn<sup>1</sup> it is impossible to differentiate the ectoplasm and endoplasm of *E. coli* when the organism is motionless, and difficult even when it is in motion, although the protoplasm is thus divided. My observations confirm his as regards this, and also as to the ectoplasm being much less refractive to light than the endoplasm, when the two can be distinguished. When distinguishable the ectoplasm presents a perfectly homogeneous appearance, it being impossible to demonstrate any structure even with the highest power objective. I have not noticed that the ectoplasm was more distinct in the larger amebae of this species than in the smaller, so that I think that it may be safely said that the ectoplasm of this parasite is difficult to distinguish at all stages of its growth. In the encysted organisms the ectoplasm is replaced by a slightly wrinkled, refractive cyst wall, which is impervious to staining solutions and evidently of very dense structure. In some instances definite layers of very refractive material may be seen to compose this wall.

The endoplasm, which constitutes the greater portion of the protoplasm, presents a finely granular appearance and an examination with high power shows that it is composed of a delicate network and minute granules which appear to float in a fluid medium inclosed in the network of fibrils. Bacteria are generally observed in the endoplasm, as well as other bodies to be described later. As the majority of *E. coli* do not show any differentiation into ecto- and endoplasm, the whole protoplasm most commonly appears finely granular

as described for the endoplasm. The very young amebae of this species appear homogeneous throughout.

When in motion the pseudopodia, formed by the ectoplasm, are scarcely to be distinguished from the endoplasm, except that they are slightly less refractive to light than is the endoplasm, and a single pseudopodium often resembles a veil-like membrane extending from some portion of the periphery of the organism, and only visible upon careful focusing.

*Nucleus*.—Almost invariably a well-defined nucleus is demonstrable in *E. coli*, situated a little to one side of the center of the organism, and possessing a thick, easily distinguished, nuclear membrane. The nucleus is large, spherical in shape, and contains a hyalin, grayish substance, embedded in which is a large amount of chromatin in the form of refractive grains and strands, together with one or more small, spherical nucleoli. The nuclear membrane is very refractive when focused upon in some lights and contains several very minute, bright, round granules which are probably chromatin. Schaudinn gave the diameter of the nucleus as from 5 to 8 microns, with which I agree. Not infrequently the nucleus is observed to be distinctly oval in shape, and the parts described cannot always be easily distinguished. When the organism is moving the nucleus tends to retain its relative position and does not change its shape under pressure as does the nucleus of *E. histolytica*. The nucleolus is generally to be distinguished, consisting of a very refractive oval or spherical body or bodies, if more than one is present, situated within the nuclear membrane.

*Vacuoles and contained bodies*.—A very large proportion of *E. coli* present a finely granular protoplasm in which no vacuoles are present; in the thousands of examples of this species of ameba that I have studied I have not observed more than one vacuole in but a very few specimens, and generally even one could not be distinguished. The vacuoles, when present, are of small size, dim in contour, and never contractile. The absence of vacuoles in this species is in striking contrast to the numerous vacuoles observed in *E. histolytica*.

In stained specimens bacteria are generally to be observed in the endoplasm and infrequently crystals of various kinds obtained from the feces. The further study of this species of ameba has convinced me that it is only very infrequently that they will engulf red blood

corpuscles, and experimentally it is almost impossible to make these organisms engulf the erythrocytes when blood is added to feces containing them, although in the case of *E. histolytica* the engulfing of red blood corpuscles is of very frequent occurrence. In the very few instances in which I have observed red blood corpuscles in *E. coli*, there has never been more than one or two, and while in a previous communication<sup>57</sup> I stated it as my belief "that the greater frequency with which red blood corpuscles are seen in *E. histolytica* depends very largely upon the fact that in amebic dysentery the feces contain much blood, whereas in infections with *E. coli* there is very little, if any, blood in the feces," I am now inclined to believe that the ingestion of blood by *E. coli* is an abnormal process occurring but very seldom, even when the feces contain much blood. This fact alone, however, does not warrant us in differentiating species, but, taken with the variation in morphology and method of reproduction, it is of some assistance in this direction.

*Motility.*—*E. coli* may be described as a sluggishly motile organism, in which this property is often absent, and when present, is of slight duration and very limited in extent. Motion is rendered possible by the extrusion of pseudopodia formed of the ectoplasm. Two forms of motion are most common; the first consists in the extrusion of the ectoplasmic pseudopodia, into which flows the endoplasm, and the consequent production of a very sluggish motion; the second form consists in the extrusion of pseudopodia from different portions of the periphery of the organism simultaneously, causing a change in the shape of the organism but no progressive motion. When progressive motion is present it is generally so slow that the organism has to be observed for some time before it can be detected.

The pseudopodia are small and rounded in contour, and are less refractive to light than is the endoplasm; sometimes they are so minute as to be distinguished with difficulty, which is never true of *E. histolytica*. I have never observed the long, finger-like pseudopodia in this organism that are so frequently observed in the pathogenic species, and it is always difficult to distinguish the boundary between the ecto- and endoplasm even when motility is most marked. The motility of this species of ameba is most apparent in freshly voided

feces, and it is never observed in feces which have stood at room temperature for more than 15 or 20 minutes.

*Staining reactions.*—The methods of staining *E. coli* have already been mentioned, Wright's method being the only one that I have found of value in differentiating this species from *E. histolytica*. With this method *E. coli*, when stained, is seen to consist of three distinct portions: The ectoplasm, the endoplasm, and the nucleus.

The ectoplasm stains very dimly, the color being a light blue, and generally appears quite structureless; in a few instances, careful examination with a high-power lens (one-twelfth) will show that this portion of the organism consists of very minute granules, so small as to appear like dust, which take the stain very slightly.

The endoplasm stains very intensely a dark blue or violet, and is seen to be composed of deeply stained, well-defined granules, some of which are of considerable size. Scattered among these granules may be seen bacteria of various kinds, and sometimes a few small crystals. If a vacuole, or vacuoles, be present, they are observed within the endoplasm, and do not contain within them any of the staining solution.

The nucleus stains a bright red or crimson, because of the large amount of chromatin that it contains. When division is not occurring the nuclear membrane is very distinct, staining more intensely than any other portion of the nucleus. Within the nuclear membrane the chromatin may be seen as short strands or spherical grains, stained a brilliant red or violet, separated by minute, unstained spaces. The nucleolus stains a very dark violet, but is often indistinguishable in stained specimens. When the nucleus is undergoing division, the chromatin may be seen arranged in two separate masses; spread uniformly throughout the protoplasm; or arranged irregularly, according to the stage of division. The extrusion of the chromatin from the nucleus at certain stages or reproduction, described by Schaudinn, is often observed in stained specimens, small groups of granules of chromatin being seen in the endoplasm of the ameba. The encysted forms, so easily distinguished in the fresh feces, cannot be studied in stained preparations, for the reason that it is impossible to stain them.

*Methods of reproduction.*—Until Schaudinn's researches but little

was known regarding the method of reproduction of the amebae in the human intestine; a few observers, working with both species, have described roughly certain reproductive phenomena, but as they were unable to distinguish the two organisms, their descriptions are naturally of but little value to the student of this subject. Celli and Fiocca<sup>30</sup> working, in all probability, with the harmless species, describe the life-cycle as consisting of an ameboid stage, a resting stage, and an encysted stage; Cassigrandi and Barbagallo<sup>32</sup> were the first to describe accurately the methods of reproduction in *E. coli*, consisting in simple division, and the formation, under certain conditions, of cysts, in which are developed eight young amebae. To Schaudinn,<sup>1</sup> however, we owe the clearest description of the methods of reproduction of this species of ameba. He found that in the liquid stools simple division was the common mode of reproduction, the nucleus first dividing, followed by division of the protoplasm, thus forming two amebae. When the stools became semi-formed or formed, he found that simple division ceased and reproduction within a cyst occurred. This process is complicated, and is best studied in a glass cell, kept at an even temperature (about 75° to 80° F.) and shielded from the light as much as possible. I have been able to follow almost every stage in the process, as described by Schaudinn, and the following description is based upon that given by Schaudinn and my own observations:

*E. coli* when about to encyst, becomes perfectly motionless, and soon, if carefully observed, is seen to develop apparently from its periphery (or ectoplasm) a very refractive hyaline membrane, at first having a single outline but gradually acquiring a double outline, and finally appearing mamillated or irregularly striated. During the development of this cyst wall, the organism has contracted, and when it is fully formed, it appears at least a third smaller than before it became encysted, thus leading us to believe that the cyst-wall is formed of materials secreted by the ameba. Within the cyst-wall is situated the protoplasm and nucleus, both of which undergo changes as development proceeds. The protoplasm, at first granular in appearance, becomes hyalin and homogeneous, the foreign bodies, such as bacteria, having been extruded during the formation of the cyst wall. After the protoplasm has become hyalin, the nucleus undergoes very



complicated changes which result in the formation of eight daughter-nuclei. The changes are briefly as follows:

The original nucleus divides into two portions, each portion being surrounded by a portion of the protoplasm, so that at this stage the cyst-wall contains within it two well-defined masses consisting of a nucleus and protoplasm. Each new nucleus now resolves itself into chromidia, a portion of which, with the remains of the original nucleus is extruded. Each mass of protoplasm now contains a small amount of chromidia, from which a new nucleus is formed; each new nucleus now divides into two, one-half of each being extruded; this process is repeated and finally ceases, two mature nuclei being the result. At this stage the two masses of protoplasm fuse, and the cyst-wall becomes much thicker. Reproduction now commences by the division of the two nuclei, two daughter-nuclei being formed, called the active and passive pronuclei; the active pronuclei fuse with the passive pronuclei, forming two synkarya, each of which divides into four, so that the cyst contains eight nuclei. Under favorable conditions the cyst-wall ruptures, and the eight small amebae, each consisting of a nucleus and a portion of protoplasm, are liberated.

It requires hours of study to follow the changes described, but it is not difficult, by studying the encysted forms in the feces at varying periods of time, to observe many of them. The mature cysts are often observed, consisting of a thick cyst-wall, a homogeneous protoplasm, in which the nuclei of the young amebae are embedded; these appear as small, spherical, very refractive bodies, in many instances very sharply outlined.

Reproduction by simple division I have frequently observed, the process being essentially as follows: The nucleus becomes elongated, the nuclear membrane appearing to become thinner and less refractive, while the minute granular contents of the nucleus (the chromatin) appear to concentrate at each pole as the elongation occurs. After the nucleus has become much elongated a constriction occurs near the center and eventually two new nuclei are formed by division, a considerable amount of chromatin being extruded into the protoplasm at the time of division. Coincident with the elongation and division of the nucleus, the protoplasm of the ameba becomes less granular, and a constriction appears, which, after the division of the nucleus,

deepens and finally becomes complete, two new amebae being thus formed. In the stained specimen the chromatin can easily be distinguished and the nuclear changes studied, but much patience is required in the search for amebae undergoing division, especially if every step of the process is to be studied.

*Relation to disease.*—While I am firmly of the opinion that the data already given regarding the presence of *E. coli* in the feces of healthy individuals for months and even years without producing symptoms of disease, is positive proof of the harmless nature of this organism, it may be of value to detail the experimental evidence we possess of this fact, evidence which it seems to me is incontrovertible.

Kartulis<sup>23</sup> experimented with the amebae obtained from the healthy intestine, by injecting them into the intestinal canal of cats, and was not able to produce any pathological lesions or symptoms. Kruse and Pasquale,<sup>25</sup> working with amebae obtained from the feces of healthy individuals, were not able to produce any of the pathologic lesions of dysentery in cats. Celli and Fiocca<sup>30</sup> use as one of their strongest arguments against the pathogenic action of amebae that they were not able to induce dysentery in animals with the amebae from healthy individuals or those from dysenteric patients. These authors were undoubtedly working with *E. coli*, as all of their experiments gave negative results. Kovacs<sup>23</sup> was unable to produce dysentery in cats with the amebae found in healthy individuals. Strong and Musgrave<sup>28</sup> were unable to produce dysentery in cats by the injection of amebae from healthy individuals, and their comment regarding their work is of interest. They say, "One of these cases which has been under our observation for several months has had these harmless amebae in his stools constantly during that time, yet he has no dysentery and no history of any, and he has no intestinal trouble. We have repeatedly injected large numbers of these non-dysenteric amebae (*amoeba coli*) while motile in the stools, into the rectum of cats, but with no effect. We have neither been able to produce dysentery with them nor any lesions of the large bowel. . . . On the other hand we have had no difficulty in producing dysentery and ulcerations of the large bowel in cats by injection of the stools or contents of liver abscesses containing motile amebae dysenteriae."

Jürgens<sup>35</sup> found that *E. coli* was not able to penetrate the normal mucous membrane of the intestine, and that it had no pathogenic action upon this membrane. He considered that this was due to the slight strength of the ectoplasmic pseudopodia, which, as has been said, is of very delicate structure, so delicate as to be almost invisible in many instances. Schaudinn<sup>1</sup> confirmed Jürgens' observation regarding the pseudopodia, and was also unable to produce dysentery or any lesion of the bowel in animals by feeding experiments or the rectal injection of material containing *E. coli*. He twice inoculated himself by swallowing the encysted forms, and numerous ameba were afterward found in the feces, but in neither instance did any symptoms of diarrhea or dysentery develop, and the amebae finally disappeared.

I have made a large number of experiments as to the pathogenic properties of *E. coli*, using young kittens as the animals experimented

upon, injecting feces containing this organism into the rectum, as well as feeding with milk containing the infested feces. I have injected into the rectum of kittens fecal material containing the encysted forms of *E. coli*, as well as the motile form, and have never been able to produce the least symptom of diarrhea or dysentery by such injections, although 50 per cent of the kittens given rectal injections of feces containing the pathogenic *E. histolytica* developed severe dysentery, of which most of them died. These injections have been repeated upon the same cat from five to ten times, and in no case has there been a diarrhea produced or any evidence of intestinal inflammation. I have repeatedly fed kittens with milk containing large amounts of fecal material with the cystic and vegetative forms of *E. coli* and in not a single instance were there produced any symptoms of diarrhea or dysentery, although these feedings were repeated at frequent intervals. In kittens fed with milk containing feces infected with *E. histolytica* over 65 per cent developed severe dysentery, as will be described later.

It would appear certain from the experiments of the various observers mentioned, and from my own, as well as the observations of the very frequent occurrence of this species of amebae in the stools in health and in diseases other than dysentery that have been detailed in previous sections of this report, that *E. coli* is a harmless parasite which is present in a large proportion of individuals in many parts of the world. The non-pathogenic character of *E. coli* is as fully proven as is the pathogenic character of *E. histolytica* and I believe that we are fully justified in accepting Schaudinn's classification as regards this common and harmless species.

#### DESCRIPTION OF ENTAMOEBA HISTOLYTICA.

*General description.*—*E. histolytica*, like *E. coli*, consists of a mass of protoplasm containing a nucleus. One and generally several vacuoles are present, which are not contractile. The shape varies with its movements, but it is always spherical when motionless. The protoplasm consists of two very distinct portions, an outer, or ectoplasm, and an inner, or endoplasm. The nucleus is situated in the endoplasm, and contains a nucleolus, which, in most instances, cannot be distinguished in the fresh specimen. The organism possesses active

motility by means of pseudopodia. Reproduction occurs by simple division and sporulation or gemmation. The following detailed description is the result of the study of many thousands of these organisms, and refers only to the morphology of the amebae as they are observed in the feces. There can be no doubt but that the morphology varies greatly upon cultures, and for this reason we can not use cultural characteristics as a basis for the differentiation of this organism from *E. coli* as it appears in the feces.

*Size.*—In the majority of instances *E. histolytica* is larger than *E. coli*, and here, as in the case of the latter organism, various authorities have given very diverse measurements, for the reason that amebae of the same species vary in size according to the period of growth at which they are observed, and because most authors have confused the two species of amebae most commonly found in the intestine of man. I am of the opinion that the size of *E. histolytica* is generally under- rather than overstated by most authorities, and from my observations I believe that *E. coli*, in the vegetative stage, is very seldom less than 10 microns in diameter, and generally much larger. When we consider that the normal red blood corpuscle averages 7 microns in diameter, it is at once apparent that an ameba of this species is very seldom observed as small as a red cell. As is well known, many amebae of this species are observed to contain red blood corpuscles, even the smaller organisms containing one or two, while it is not uncommon to observe organisms containing six or eight red cells, without being distended by them, thus proving that the average size of this amebae is considerably in excess of that of a red blood corpuscle. I have often seen amebae of this variety containing from 20 to 30 red blood corpuscles, which would make their diameter at least 50 microns, and I have repeatedly seen individuals measuring as much as 60 to 70 microns. As the result of my observations I believe that the great majority of individual *E. histolytica* measure at least 35 microns in diameter, a much larger average measurement than that of *E. coli*. These remarks do not apply to the young, free spores of this species, which measure about 5 microns in diameter, and are easily distinguishable from the vegetative form of the organism.

The size of *E. histolytica* is of importance both from a diagnostic and etiologic standpoint. As I have stated, while the size of *E. coli*

approximates that of the pathogenic species, I have never seen it equal the larger specimens of *E. histolytica* which are often observed, and the average is much smaller. If this fact be remembered, together with the differences in morphology, it is not a difficult matter to differentiate the two species. Some authorities have gone so far as to found the species distinction entirely upon the size of the amebae, the larger being classed as pathogenic, the smaller as non-pathogenic. With such a classification I cannot agree. Careful examination of the feces in cases of amebic dysentery will show that amebae of large size are not always present, while in other cases both large and small amebae are found, belonging to the pathogenic species, so that the most we can say regarding size in the differentiation of these two species is that, in the vast majority of instances, *E. histolytica* averages larger than *E. coli*.

*Shape*.—The shape of the organism varies with its movements, but when it is at rest it is spherical or slightly oval. Kartulis,<sup>13</sup> in his description of the amebae occurring in amebic dysentery, states that when at rest the organism is oval, but observation will demonstrate that the spherical shape is altogether the more common. When in motion the organism presents most extreme variations in shape, owing to the changes in the form of the pseudopodia.

*Color*.—I have spoken of the peculiar dull-grayish color of *E. coli*; as a rule *E. histolytica* is almost colorless, the ectoplasm being perfectly so, appearing like a piece of ground white glass. The endoplasm is of a very light-gray color in most instances, but when blood is present in the feces, there is often observed a slight greenish tint in this portion of the organism. This color I believe to be due to hemoglobin liberated during digestion of red blood corpuscles by the ameba; this explanation has been questioned by some authors, but there can be no doubt that *E. histolytica* does engulf and destroy these cells, and anyone can convince himself of this fact who will spend the time necessary to demonstrate it. I have time and again watched this ameba engulf red blood cells, and traced the process of digestion, the cells gradually losing their color and breaking up into fragments. I have repeatedly seen an ameba cross the microscopic field and engulf a red blood cell, just as the leucocytes engulf the malarial plasmodia; it would indeed be strange if such a manifest example of phagocytic activity did not

result in the destruction of the engulfed body, and I believe that this phenomenon proves that *E. histolytica* is capable of destroying red blood corpuscles, which is not true of *E. coli*, for in the few instances in which I have observed red blood cells within this organism, I have never seen any evidence that the ameba was able to destroy them.

*Protoplasm*.—The appearance of the protoplasm in *E. histolytica* varies somewhat with the age of the organism. In the small or young amebae, the protoplasm is finely granular in appearance, and unless the organism is in motion it is very seldom that the ectoplasm and the endoplasm can be distinguished. The nucleus is, as a rule, invisible, but when visible, is situated to one side of the center of the endoplasm. As the organism enlarges the ectoplasm and endoplasm become differentiated, and in the large amebae these two portions of the protoplasm may be differentiated, in many instances, even when the organisms are motionless. In the moving organism the distinction is always very clear and unmistakable.

The ectoplasm comprises about one-third of the protoplasm and appears perfectly hyalin and glass-like, although an examination with the highest power lenses demonstrates that it is composed of some dense material and innumerable very minute granules. This portion of *E. histolytica* is very refractive to light, much more so than the endoplasm, the opposite of which is true in *E. coli*. One has but to compare the firm-appearing, well-defined ectoplasm of *E. histolytica* with the delicate, indefinite ectoplasm of *E. coli*, to be convinced of the truth of Schaudinn's and Jürgen's assertion that the secret of the pathogenic action of *E. histolytica* lies in the ability of the ectoplasm to penetrate the mucous membrane of the intestine, a property which the ectoplasm of *E. coli*, by reason of its delicate structure, does not possess.

The endoplasm, comprising about two-thirds of the body of the fully developed organism, is light grayish in color, and is composed of granular material, the granules being of considerable size, and more or less refractive. The endoplasm of this species appears much more granular and coarser than the endoplasm of *E. coli*, and is not as refractive to light.

While the granules comprise the greater portion of the endoplasm of this ameba, one, and generally more than one, vacuole, is always

present, and in many of the largest amebae, the entire protoplasm appears vacuolated, a change which is probably degenerative in character. Besides the vacuoles, the endoplasm may contain red blood cells, bacteria, crystals, and peculiar oval bodies, the nature of which will be explained later.

*Nucleus*.—A nucleus is, of course, always present in *E. histolytica* but it is generally very difficult to distinguish, in contradistinction to *E. coli*, in which nearly every organism shows a distinct and easily observed nucleus. When it can be seen, the nucleus is situated, as pointed out by Schaudinn, eccentrically in the endoplasm, and it is often seen flattened against the boundary between the ecto- and endoplasm. It contains but little chromatin compared to the nucleus of *E. coli*, is circular in shape, and may contain a definite nucleolus. The nuclear membrane is very indistinct, and the structure of the nucleus difficult to ascertain. When the organism is in motion the nucleus continually changes its relative position, unlike the nucleus of *E. coli* which tends to retain its relative position in the endoplasm. The nucleus of this species is not very refractive, is colorless, and the nucleolus, when it can be distinguished, is less refractive than the nucleus and situated to one side of the center of that body. The size of the nucleus varies with the stage of growth of the organism but averages about 5 to 6 microns when fully developed. In the large amebae the nucleus is not visible, as a rule, because during reproduction by sporulation the nucleus is distributed, and thus becomes invisible in all but stained specimens. Where reproduction occurs by simple division the nucleus is always visible, situated near the center of the organism.

*Vacuoles and contained bodies*.—The endoplasm of *E. histolytica* always presents one or more vacuoles. In the smaller amebae only one is visible but in the fully developed organisms the vacuoles may vary in number from one to ten, or even more. When only one vacuole is present it is of large size, but when more than one is present, the size varies. In certain organisms the entire protoplasm appears to be replaced by vacuoles, and in such I have considered the process as of a degenerative nature. In many instances the vacuoles contain within them dissolved hemoglobin, thus having a greenish tint, bacteria, very small crystals, and peculiar, very refractive granules, in

active motion. The vacuoles are never contractile, and are perfectly spherical in shape when the organism is not in motion. When in motion the vacuoles change position with the movement of the endoplasm.

There can be no doubt but that *E. histolytica*, unlike *E. coli*, is normally phagocytic for the red blood corpuscles of man. This can be easily demonstrated by adding blood to feces containing these organisms and watching the result. Red blood corpuscles are very commonly seen within this organism, not alone because the feces in amebic dysentery frequently contains blood but because this ameba is normally a red corpuscle destroyer. In those cases of amebic dysentery in which both *E. histolytica* and *E. coli* are found in the feces, and such cases are far from uncommon, it is but very seldom that *E. coli* is seen to contain red corpuscles, although almost every specimen of *E. histolytica* may be filled with them. In the great majority of cases where these two species occur together, not a single specimen of *E. coli* will be seen to have engulfed red corpuscles, while numerous examples of such action by the pathogenic ameba can be demonstrated.

Generally not more than two to six red cells are observed in the endoplasm but it is not rare to see *E. histolytica* containing so many red cells that but little of the structure of the ameba can be distinguished; such amebae, if kept at the body temperature and carefully observed, will be seen to gradually break up these red cells, the hemoglobin coloring the organism a light green. The cells are not extruded but are very evidently digested, and the extrusion of red cells, which sometimes occurs, is an abnormal process induced by unfavorable changes in the environment, as, for instance, observation of the amebae without the use of a warm stage maintained at the right temperature. Besides red blood corpuscles, bacteria of various kinds, pigment granules, and crystals of various kinds are observed within the endoplasm of *E. histolytica*, as well as small oval bodies, which will be described in speaking of the methods of reproduction of this organism.

*Motility.*—In recently passed specimens of feces from cases of dysentery *E. histolytica* possesses very active motility, and if a warm stage be used these organisms preserve their motility for a long time. There can be no doubt but that this species of ameba is much more



actively motile than is *E. coli*, and that this is a valuable distinguishing feature between the two organisms. Musgrave and Clegg<sup>56</sup> do not agree with this statement and attempt to disprove it by comparing the rate of movement of various amebae upon culture plates with the movement observed in the feces. Such a method of comparison is of little value, as the environment is not the same, neither were the authors mentioned using pure cultures of *E. coli* or of *E. histolytica*, so far as their experiments indicate. As I already pointed out we cannot draw conclusions regarding the amebae observed in feces by what occurs during the artificial cultivation of amebae from various extraneous sources.

In *E. histolytica* motility may be very marked and when present is always more marked than the most active motility seen in *E. coli*. The mere extrusion of the pseudopodia is more rapid, even though progressive motion may not occur, while the division between the ectoplasm is very distinct. Three forms of motility may be distinguished; active progressive motion, the extrusion of pseudopodia without resultant progression, and movements of the endoplasm.

The character of the progressive motion of *E. histolytica*, in freshly passed feces, is generally rapid for ameboid organisms, and especially so when it is compared to the progressive motion of *E. coli*. As the feces containing the amebae loses the heat imparted to them by the body, the amebae gradually lose their progressive motion, and finally become motionless. The organism advances by throwing out pseudopodia, composed entirely of the clear, glass-like ectoplasm, into which flows the endoplasm. In cooled specimens the motion may be so sluggish as to require careful and prolonged observation to distinguish it, but in specimens kept at the temperature of the body *E. histolytica* exhibits a comparatively rapid progressive motion. The pseudopodia of this species of ameba vary considerably in size and general appearance, but are always larger, more distinct, and more commonly observed than in *E. coli*. The shape varies from a broad, rounded mass of ectoplasm to long, finger-like processes, with rounded extremities. I have never observed pointed pseudopodia in this species in the stools, although such forms are said to occur upon cultures. The long slender pseudopodia are most frequently observed in those organisms which are moving rapidly, while the broader and

more rounded pseudopodia occur in ameba which are more sluggish in their motion. When first extruded the pseudopodia are always hyalin in appearance, being composed entirely of ectoplasm, but the endoplasm quickly flows into them, and a granular appearance is thus produced; in very rare instances amebae of this species are observed in which the motion is so rapid and continuous that the distinction into ectoplasm and endoplasm cannot be made, but if such organisms be observed for a sufficiently long time, motion becomes slower and then the two portions of the protoplasm can be differentiated. I have spoken of the very gradual flowing in of the endoplasm into the pseudopodia of *E. coli*, this phenomenon occurring so slowly, and the two portions of the protoplasm of that organism being so similar in appearance as to render the observation of it almost impossible. This is not so in *E. histolytica*. In this organism the flowing into the ectoplasmic pseudopodia of the endoplasm occurs very rapidly as a rule, it often appearing as though the periphery of the endoplasm ruptured, thus allowing the remainder of this portion of the protoplasm to rush into the pseudopodia. In many instances the pseudopodia appear constricted near the boundary of the endoplasm, and when this occurs the endoplasm may be seen to pass slowly through the constriction, the contents, as the nucleus, vacuoles, and red blood corpuscles, being compressed as they pass through the narrowed portion. When motility is marked, as in fresh specimens of feces, the amebae are often seen to progress in a definite direction, as toward a blood corpuscle, or other substance, or to cross the microscopic field without cessation of progressive motion; in other instances motion will occur in one direction, quickly followed by progression in nearly the opposite direction, so that it may be many minutes before the ameba will pass out of the field of a one-sixth-inch objective.

In specimens which have been exposed to room temperature for some time, the second form of motion is frequently observed. This consists in the active extrusion of pseudopodia, but no progressive motion, the processes of ectoplasm being continually projected from the periphery of the ameba, and as quickly withdrawn. In such instances the endoplasm does not flow into the pseudopodia, as a rule, and when it does, a new pseudopodium is projected from some other portion of the periphery of the organism, thus neutralizing what would

otherwise result in slight progression. This form of motility is only observed in amebae which are exposed to unfavorable conditions, as lowered temperature, and mild solutions of certain chemical substances.

In rare instances, what may be called an intraprotoplasmic form of motion is observed, produced by currents in the protoplasm of the ameba. In such instances the contents of the protoplasm are seen to be in motion in a circular manner within the ectoplasm, the motion being most apparent toward the periphery of the organism. The motion may be slow or so rapid as to be hardly distinguishable; the nucleus, if visible, the vacuoles, red blood cells, bacteria, and crystals being whirled about within the protoplasm of the ameba. An undulatory motion of the border of the organism is often present along with this movement of the protoplasm.

As to the significance of this form of motion we are ignorant. It is very similar to that occurring in *Balantidium coli* when encystment begins, preparatory to reproduction, but although such ameba have been observed for hours, encystment does not occur, but, on the contrary, the intraprotoplasmic motion ceases, and progressive motion may be resumed.

*Staining reactions.*—With the modification of Wright's stain described, *E. histolytica* presents definite staining reactions serving to distinguish it from *E. coli*. In the larger organisms the ectoplasm stains very intensely, while the endoplasm stains dimly, the opposite of which is true in *E. coli*; in the smallest organisms this distinction cannot be as easily made, but every specimen of feces containing this species of ameba will present numerous individuals in which this distinctive staining of the ectoplasm and endoplasm will be found. The nucleus of this ameba stains very poorly as compared to that of *E. coli*, on account of the small amount of chromatin present in it, as well as from the fact that in many of the ameba the nucleus is undergoing division prior to sporulation. With Wright's stain the ectoplasm stains a very intense blue or violet, the endoplasm a light blue, and the chromatin of the nucleus a delicate pink or red. The staining reaction of the forms undergoing reproduction will be described in the next section. As I have already stated, it is difficult to properly stain these organisms for the purpose of morphological study, and many

preparations will have to be examined before one can expect to obtain suitable material for this purpose.

*Methods of reproduction.*—To Schaudinn we owe the first accurate description of the method of reproduction of *E. histolytica*, upon which he based very largely his classification of the species, and which the opponents of this classification have never disputed. The difference in the method of reproduction of *E. histolytica* and *E. coli* alone amply suffices to establish the two species.

*E. histolytica* reproduces in two ways: by simple division and by a process of budding or sporulation. Simple division of an ameba into two parts occurs in the feces under favorable conditions, and the process can frequently be observed if a warm stage is used and the organisms be watched for a sufficient period of time. It does not differ from the process already described for *E. coli*, the nucleus first dividing, followed by the division of the protoplasm, and the formation of two motile amebae.

Prior to Schaudinn's observations regarding the method of reproduction of this species of ameba I published<sup>57</sup> a description of the organism in which I described what I considered might be spores which were situated in the endoplasm and stained dimly with methylene blue. In concluding this paper I said:

There occur in all but the degenerative forms of ameba small, round, or oval, dimly stained areas, uniform in appearance, and most numerous in the large, full grown forms, being entirely absent in the vacuolated shells of amebae. These areas resemble similar areas in stained segmenting malarial plasmodia, and which are in them due to the young spores. Reasoning from analogy it may be that these areas in the amebae are also spores.

Schaudinn confirmed my opinion regarding these bodies. He found that under certain conditions nuclear division does not occur as in simple division but that the nucleus distributes its chromatin to the protoplasm, while the remainder of the nucleus is absorbed or extruded. This form of nuclear distribution only occurs when conditions arise in the host (man) unfavorable to the vegetative existence of the organism, and marks the first stage in the evolution of the resistant form or spore. The distributed chromatin gradually collects in small oval masses toward the periphery of the ameba, and finally becomes situated in the ectoplasm, from which the masses of chromatin, each surrounded by a portion of protoplasm, are separated, and

become free as spores. These spores Schaudinn regarded as the infective stage of the organism and proved that they were capable of producing dysentery in cats.

I have been able to confirm Schaudinn's description of this mode of reproduction and the following description is the result of the study of this process as observed in living amebae and in stained specimens.

It is not infrequent to observe amebae of this species in the feces, in which the protoplasm is seen to contain numerous brightly refractive granules and minute rod-like bodies which almost fill the organism. These bodies are the chromidia, which have been liberated in the protoplasm by the breaking-up of the nucleus. Other amebae are seen in which these refractive particles are collected into small clumps, arranged irregularly in the protoplasm or about the periphery, even distending the ectoplasm. This appearance is due to the grouping of the chromidia and the collection of these groups at the periphery, just before their liberation as spores. The final stage consists in the separation of these masses of chromidia, together with a small amount of protoplasm. Since the publication of my original papers upon this subject I have been so fortunate as to be able to follow this method of reproduction in its entirety in living specimens of *E. histolytica*, and thus to confirm Schaudinn's original description. In the feces of cases of amebic dysentery, especially in those cases in which by reason of the natural resistance of the system, or because of therapeutic measures, an unfavorable environment has been produced, the spores of *E. histolytica* occur in enormous numbers, and may be easily studied. They are round or oval in shape, have a yellowish membrane, while the contents appear homogeneous. They measure from 3 to 6 microns in diameter, the average being about 4 microns. On account of their yellowish or brownish color they resemble red blood corpuscles, under a low power objective, but can be easily differentiated from them with a one-sixth-inch objective. In stained specimens of feces containing this species of ameba, this method of reproduction can be traced by examining a large number of preparations at various periods of time. The larger amebae should be selected for study, and if Wright's method be used, the following forms illustrating various stages in the process will be observed:

1. Amebae in which the chromatin of the nucleus is stained a light

pink, and is arranged in the form of rods and granules. Compared with *E. coli* the chromatin is small in amount and does not stain so intensely.

2. Amebae in which the chromatin is partly within the nucleus and partly outside, somewhat increased in amount, and staining more intensely. This form illustrates the beginning of the distribution of the chromidia to the protoplasm and the destruction of the nucleus.

3. Amebae in which the chromatin is distributed in very faintly stained grains and granules throughout the protoplasm, and the original nucleus has entirely disappeared.

4. Amebae in which the chromatin rods and granules are collected into small clumps situated in the endoplasm and near the periphery of the organism in an irregular manner. These organisms are almost ready to sporulate.

5. Amebae in which some of the masses of chromatin are arranged in the ectoplasm causing it to project slightly, and in which one or more masses may be partially separated from the parent ameba. These various forms are illustrated in Plate 3.

In some amebae an unstained area may be seen surrounding the masses of chromatin and dimly stained protoplasm, thus showing the outline of the spore. It will thus be seen that in specimens stained by the method mentioned every stage of the process or reproduction described by Schaudinn may be observed. The spores I have not been able to stain. After they are liberated the resistant membrane surrounding them is formed and this effectually prevents the action of the staining solution. They do not in the least resemble the encysted forms of *E. coli*.

From the observations of Schaudinn, which I have confirmed in full, I conclude that reproduction in *E. histolytica* occurs in two ways: by simple division or fission and by the process of sporulation which I have described. The latter method of reproduction definitely differentiates this species from *E. coli*, and this fact, taken with the occurrence of *E. coli* in health and in diseases other than dysentery, and the experimental evidence of the effect of the two species upon susceptible animals, certainly proves beyond doubt the truth of Schaudinn's classification.

*Relation to disease.*—At the present time almost all authorities are

agreed in believing that a certain form of dysentery is caused by amebae, for it seems to me that if there is one fact absolutely proven in pathology, it is that amebic dysentery is due to the pathogenic action of the amebae invariably associated with the lesions of that disease. I shall not consider here the great mass of experimental evidence that has justified this belief, but shall consider only the evidence proving that *E. histolytica* is the species concerned in the etiology of this form of dysentery. I have already described the negative results obtained by Schaudinn and myself in experiments upon kittens with *E. coli*, experiments which definitely prove that this species has no pathogenic action upon these animals, and which can easily be repeated by anyone interested in the problem of amebic infection. I shall now describe certain experiments which show, it would appear, that *E. histolytica* is capable of causing in kittens all the lesions of amebic dysentery observed in man, and that no other organism occurring in the feces with the amebae possesses like power when separated from them. Schaudinn experimented upon cats and concluded that, in feeding experiments, the spores only of *E. histolytica* were capable of causing dysentery in these animals, and he therefore regards the spores as the infective agents in amebic dysentery.

One of Schaudinn's experiments, showing the infectivity of the spores, was as follows: He dried, in the air, the feces from an amebic dysentery case in which numerous spores were demonstrated upon microscopic examination. After thorough drying enough water was added to make about 20 preparations, using a cover-glass of 18 × 22 mm. These preparations were all examined carefully for *E. coli*, or its encysted stage, and were demonstrated to be free from this species of ameba, but the spores of *E. histolytica* were present in large numbers. The cover-glasses were removed from 10 of these preparations, the material washed off, and enough distilled water added to make 1 c. c. This was fed to a young, healthy cat in milk and upon meat. Upon the evening of the third day after feeding, the cat passed some bloody mucus, which, upon examination, was found to contain large numbers of *E. histolytica* in the vegetative stage of development. The cat died upon the afternoon of the 14th day, and section of the intestine showed the characteristic ulcerations. Another experiment which Schaudinn believes demonstrates that only the spores of *E.*

*histolytica* are able to produce infection, is as follows: A cat was fed upon material containing the vegetative stage of this organism only, and observed for a period of four weeks, during which time she remained healthy, and an examination of her stools at the end of this time demonstrated the absence of amebae. She was then fed with material containing only the spores of *E. histolytica* with the result that after an incubation period of six days she developed dysentery with bloody stools, which contained numerous amebae of this species. Death occurred at the end of two weeks and the intestine presented the characteristic lesions of amebic dysentery.

These experiments of Schaudinn throw a flood of light upon the interpretation to be given the negative results of other observers. Those who have obtained only negative results in their experiments were undoubtedly working with the non-pathogenic *E. coli*, while those who have obtained only a certain proportion of positive results in working with *E. histolytica* probably used feces in which the spores of this organism were absent in those cases in which they obtained negative results.

*Personal observations.*—I have already detailed the negative results I obtained in kittens by feeding experiments and rectal injections with feces containing the vegetative and encysted stages of *E. coli*, and I have repeated these experiments, using the feces of dysenteric cases, in which both the vegetative stage, and the spores of *E. histolytica* were present, with the result that in 65 per cent of the kittens experimented upon by feeding, typical amebic dysentery developed, and in 50 per cent of those in which rectal injections were used the same result was obtained. Control tests were made with the bacteria occurring in the feces, and with feces containing *E. coli*.

*Rectal injections.*—Half-grown kittens were used in these experiments, about 5 c. c. of feces containing the ameba being injected into the rectum. This is the method which has been employed by most investigators of this subject. I have found that 50 per cent of the kittens so injected developed dysentery. The negative cases may be explained by the absence of the spores of *E. histolytica* from the stools, for while motile amebae were present in all of the material used we, were not certain of the presence of the spores as at the time of the



experiments I was inclined to believe that the vegetable stage of this ameba could produce the infection.

In dysentery produced by the rectal injection of the infective material, the lesions tend to be localized in the rectum and are not so general or so severe, as a rule, as when the infection is acquired through the mouth. The incubation period varied from six days to nearly two weeks, and was generally longer than in the feeding experiments. The lesions produced were typical of those occurring in amebic dysentery in man, and varied in extent and severity with the length of time the infection lasted.

As an example of the character of the lesions produced by the rectal injections of material containing *E. histolytica*, the following autopsy report is given of a kitten injected October 19 and killed November 21, the first evidence of infection having appeared about October 30.

*Kitten 1.*—Body that of a half-grown kitten, very greatly emaciated. The abdomen is greatly distended with gas. The mucous membrane of the anus appears swollen and a considerable amount of blood-stained mucus is adherent to it. The sub-cutaneous fat has almost entirely disappeared and the muscles appeared dry and atrophied. The pleural cavities are free from fluid and the lungs appear normal. The heart is greatly congested and contains red clots in all the chambers. The liver is hypertrophied, deeply congested, and marked albuminoid degeneration is present but there is no trace of abscess formation. The kidneys are congested and upon section present the usual lesions of an acute parenchymatous nephritis. The omentum contains a small amount of fat and is not inflamed. The bladder is filled with urine.

The intestines are greatly dilated with gas and fluid. Upon external examination the large intestine appears swollen, is grayish in color, with small, darker colored areas scattered along it. Upon opening the large intestine the mucous membrane of the rectum is found considerably swollen and inflamed, but no ulcerations are present. Above the rectum for a distance of about 10 cm. the mucous membrane is very much swollen and edematous, bright red in color, and between the folds a considerable amount of pus can be seen. For a distance of about 4 cm. from the upper end of the large intestine, the mucous membrane is inflamed, being red, swollen, and edematous. In this area there are numerous ulcerations, covered in with bloody mucus; they are small size, somewhat irregular in shape, and extend, in most instances, to the sub-mucosa, although there are a few which extend to the muscular coat of the intestine; the edges are undermined and many of the ulcers are covered with necrotic tissue, brownish yellow in color, which has to be removed in order to expose them. A few of the ulcers communicate beneath the mucous membrane. The small intestine shows a rather severe acute enteritis and the stomach an acute gastritis.

This case was unusual in that the greater number of the ulcerations occurred near the ileo-cecal valve, while the rectum escaped, whereas in other successful experiments by rectal injection, the lesions were

almost confined to the rectum and the intestine for a short distance above the rectum.

The amebae, actively motile, were demonstrated in smears made from the intestine in this kitten, being most numerous where the lesions were most severe and least numerous in that portion where no ulcerations were present, but every part of the large intestine showed infection with *E. histolytica*. Smears made from the small intestine, immediately above the valve, did not show any amebae.

The clinical symptoms present in this case were those observed in all of the kittens that developed dysentery, and were similar to those occurring in man. The first symptom was invariably diarrhea, the stools being frequent, at first free from blood, but soon becoming bloody and filled with mucus, while numerous amebae were present in them. Emaciation occurred rapidly, and some fever was generally present. After persisting for several days, the diarrhea, in this kitten, ceased and a period of constipation intervened, in which two or three days passed without a bowel movement, but after a week the dysenteric symptoms recurred, and from that time until the animal was killed, the bowel movements varied in number from six to ten a day, and the animal became almost a skeleton. *E. histolytica* could always be demonstrated in the feces after the initial symptom of diarrhea appeared.

*Feeding experiments.*—While rectal injection of infective material results in the production of dysentery in 50 per cent of the kittens I have experimented with, the most successful results have been obtained by feeding kittens with infected fecal material. In this way I have produced typical dysentery, amebic in type, in 66+ per cent of the kittens experimented upon (8 out of 12) and have demonstrated *E. histolytica* in the feces of all and in sections of the diseased intestines.

My method of feeding was as follows: The kittens were starved for 24 hours, and at the end of that time were given milk containing about 5 c. c. of the infected feces, containing motile amebae of this species and their spores; the animals do not object to taking the mixture, and an almost equal amount of feces may be mixed with the milk without the kittens appearing averse to eating it. After the animals were fed they were observed carefully and the symptoms noted.

In all the successful cases the symptoms consisted in diarrhea with the passage of blood-stained, mucous stools, containing multitudes of motile *E. histolytica*, rapid emaciation, loss of appetite and strength, severe tenesmus, the kittens appearing much distressed while voiding the feces, and finally death from exhaustion.

The period of incubation varied considerably, the shortest being seven days, the longest eleven days; most of the animals showed diarrhea, with the passage of blood-stained stools, by the 8th day, so that the period of incubation is shorter by this method than by the use of rectal injections. Short periods of constipation were observed in two of the animals, lasting a day or two, but were always succeeded by profuse diarrhea with the passage of almost pure blood and mucus.

As illustrating the pathological lesions found in these animals, and produced by feeding with material containing *E. histolytica* the following autopsy records are inserted:

*Kitten 3.*—This kitten was fed once with feces containing *E. histolytica* and seven days later developed diarrhea, the feces containing blood and mucus, as well as numerous motile amebae. At the end of two weeks it died, having presented severe symptoms of amebic dysentery during this time.

*Autopsy.*—Body that of a half-grown kitten, very greatly emaciated. Subcutaneous fat entirely absent, and muscles dry and much atrophied. The abdominal cavity is free from fluid and the intestines appear normal externally. The pleural cavities are free from fluid and the heart and lungs appear normal. The liver is brownish red in color externally, with irregular yellow motlings. There is a small abscess present at the dome of the right lobe, measuring 0.25 cm. in diameter, showing very distinctly through the capsule of the organ. Upon section of the liver the cut surface appears greatly congested, the lobules are distinct, and no abscesses are found other than the one mentioned. The gall bladder appears normal. The kidneys appear enlarged and congested and upon section show an acute congestion, with some thickening of the cortex. Externally the large intestine appeared slightly, if at all, congested, although the walls were markedly thickened. Upon opening the large intestine it was found filled with fecal material mixed with a large amount of pus, and blood-stained mucus. About 1 cm. from the anus, which was blood-stained and covered with mucus, there was an area measuring 4 cm. in length, presenting the typical lesions of amebic dysentery, as they are observed in man. The entire mucous membrane was swollen, congested, and edematous. Numerous nodular areas projected into the lumen of the intestine, which, when incised, were found filled with a glairy material containing hundreds of *E. histolytica*. There were also numerous ulcerations, more or less irregular in shape, with thickened and undermined edges; many were covered in with necrotic tissue, which, upon being removed, showed that the floor of the ulcer was formed by the muscular coat of the intestine. Many of these ulcers communicated with one another beneath the mucous membrane, and most of them had penetrated to the muscular coat. The remainder of the large intestine presented numerous ulcera-

tions, typical of those seen in the intestine of patients who have died of amebic dysentery. The lesions were most marked just below the ileo-cecal valve, where large areas of the mucous membrane had been destroyed, the muscular coat of the intestine being exposed.

*Kitten 5.*—This kitten was fed with milk containing *E. histolytica* several times before dysentery developed. The period of incubation was eight days from the date of the last feeding, but from that time, until it was killed, three weeks afterward, the animal presented the symptoms of amebic dysentery, there being gradual loss of appetite, emaciation, and a diarrheal discharge, containing blood and mucus, with numerous motile *E. histolytica*.

*Autopsy.*—Body that of a half-grown kitten, much emaciated. Subcutaneous fat entirely absent and muscles much atrophied. The pleural cavities were free from fluid and the lungs and heart appeared normal save for congestion. Upon opening the abdominal cavity the small intestine appeared congested externally. The liver is hypertrophied and greatly congested. The kidneys are congested and enlarged and upon section showed the lesions of an acute parenchymatous nephritis. The large intestine was dark gray in color externally, and was considerably thickened, especially toward the rectum. Upon opening the intestine it was found to contain much fecal material, mixed with blood, mucus, and pus. Commencing at the rectum and extending for about half the length of the large intestine, the mucous membrane was greatly swollen, bright red in color, and contained numerous ulcers. The majority of the ulcers were spherical in shape, the edges were undermined and greatly thickened, and many were covered in with necrotic tissue. Upon removing this necrotic material the base of the ulcer is found to be formed by the muscular coat of the intestine. The ulcers present were typical of the amebic ulcerations seen in the intestine of man in every respect. The remainder of the large intestine was black in color and gangrenous, the mucous membrane having been almost entirely destroyed, exposing the muscular coat throughout this portion of the intestine. About 4 cm. below the ileo-cecal valve there was a small perforation measuring about  $\frac{1}{8}$  cm. in diameter.

From the protocols of the autopsies given I believe that it is evident that *E. histolytica* produces in kittens the typical lesions of amebic dysentery as they are observed in man. All of the kittens experimented upon were examined prior to the experiments as to the presence of amebae in their feces, and all were found negative in this respect. The examination of sections of the intestines presented the same microscopic pathology observed in sections from dysenteric intestines from man and the amebae could be demonstrated in the tissues. It is a significant fact that the amebae observed in the feces and in the intestine of every kitten in which dysentery resulted from either the injection of infected material, or from the feeding of such material, presented the morphological characteristics of *E. histolytica*, no organisms answering to the description of *E. coli* being observed.

*Control experiments.*—That *E. histolytica* produced the lesions of

dysentery observed in the infected kittens, and not the bacteria occurring with the amebae, was conclusively proven by using cultures obtained from the feces for feeding and injections in the same manner in which the feces containing the amebae were employed. Mixed cultures of all the bacteria that could be cultivated were used in the controls and no evidence of dysentery was produced. While there were probably bacteria present that could not be cultivated, I think that the conclusion is justifiable that *E. histolytica* is the cause of amebic dysentery. Further proof of the etiological relation of this species of ameba to the lesions produced is found in the fact that in the abscess of the liver which occurred in one of the infected kittens the pus was sterile save for a few of the amebae, and these organisms were also demonstrated in sections made of the abscess.

As I have already noted, the controls made with feces containing *E. coli* resulted negatively in every case.\*

#### DIFFERENTIAL DIAGNOSIS OF ENTAMOEBAS HISTOLYTICA AND ENTAMOEBAS COLI.

The differential diagnosis of *E. histolytica* and *E. coli* rests upon the study of their morphology and of their methods of reproduction. From a practical standpoint the diagnosis must be made from differences in the appearance of the two species as they are observed in the feces, and such a diagnosis can be made when material is available and the two organisms can be studied together. I am firmly of the belief that anyone who has so studied these organisms will be forced to admit that they vary greatly in morphology, so much so as to render a differential diagnosis possible in every case. I have demonstrated to several investigators the differences in the morphology of the two species, and, while at first they had not accepted the plurality of species, careful study convinced them of the truth of Schaudinn's classification. In discussing the subject of the differential diagnosis of the amebae infecting the intestine of man in a previous paper, I said:

I am convinced that many cases have been diagnosed amebic dysentery, which in reality presented the harmless *Entamoeba coli* in the feces, this organism being mis-

\* From the results obtained in the experiments of Schaudinn and myself, it is evident that the term "amebiasis" used by some recent authors to indicate amebic dysentery, is incorrect, and should be discarded. The term might be used in a general sense to indicate the presence in the intestine of both species of amebae but its use as a synonym of amebic dysentery cannot be recognized by those who accept the classification of amebae into a pathogenic and non-pathogenic species.

taken for *Entamoeba histolytica*. This mistake might easily be made in patients suffering from acute enteritis, in which it is more than probable that the majority would present *Entamoeba coli* in the feces, and this fact undoubtedly explains the numerous instances of so-called amebic dysentery with rapid and complete recovery.

From my experience there is no disease so resistant to treatment and in which a prognosis is so discouraging as amebic dysentery. Everyone is familiar with the fact that amebic dysentery recurs even after long periods of time, and it is very important, both to the patient and the physician, to know absolutely that the disease being treated as amebic dysentery is in reality due to *Entamoeba histolytica*, and that *Entamoeba coli* has not been mistaken for this organism.

During nearly four years' further study of the amebae occurring in the intestine of man, I have seen no reason to change my belief as expressed in the quotations given; indeed, further experience has only strengthened it, and while I have been able to add little to my original description of the morphology of these organisms, I am convinced that a differential diagnosis of *E. histolytica* and *E. coli* can be made with little difficulty upon morphological data alone.

The following are the principal differential features between two species as they are observed in freshly voided feces. It should be remembered that the differential diagnosis is arrived at from a consideration of *all* the data given, and not, as a rule, from the presence of a single feature. It is too much to expect that every individual organism will present all the points of difference enumerated, but many of them will, and in those that do not, enough will be present to render a diagnosis possible. To illustrate: not every large, motile ameba, without a distinct nucleus, is an *E. histolytica*, but if to these characteristics be added very marked motility, a clearly differentiated and highly refractive ectoplasm, and, perhaps, the presence of red blood corpuscles within the endoplasm, we may rest assured that we are dealing with *E. histolytica* and not *E. coli*. Bearing in mind, then, that our differential diagnosis must depend upon the presence of several morphological features, rather than any one, the following are the chief points in which *E. histolytica* differs from *E. coli*, as these organisms are observed in the feces.

*Size*.—As a rule *E. histolytica* is considerably larger than *E. coli*, but we cannot base a differential diagnosis upon size alone. This factor is only of value when other morphological data are considered with it, except in rare instances in which the very large size of the

ameba is enough in itself to enable us to designate the species, for *E. coli* never reaches the size of the largest *E. histolytica*.

*Color.*—The color of the two organisms is of some assistance in distinguishing them. In *E. coli*, the ecto- and endoplasm are both grayish in color and there is never observed the greenish color which is not uncommon in *E. histolytica*; in the latter species the ectoplasm is always colorless and hyaline in appearance, while the endoplasm is grayish or greenish. These observations apply only to the amebae as they appear in the stools.

*Protoplasm.*—The very marked distinction between the ectoplasm and endoplasm in *E. histolytica* is one of the most important features which differentiate this species from *E. coli*. This distinction can always be made in the motile amebae and generally in those which are not moving, the ectoplasm being visible at some portion of the periphery of the organism. In *E. coli* the ecto- and endoplasm can with difficulty be distinguished in the moving organisms, but never in the quiescent ones, and there is never present in this species the glass-like, perfectly hyaline appearance of the ectoplasm invariably observed in *E. histolytica*.

In *E. histolytica* the ectoplasm is very strongly refractive to light, much more so than the endoplasm; in *E. coli* the endoplasm is most refractive, but neither the ectoplasm or the endoplasm is as refractive as is the ectoplasm of *E. histolytica*.

*Nucleus.*—In *E. histolytica* the nucleus is generally invisible, and when visible is situated near the periphery of the organism, contains but little chromatin, is small, and possesses a very poorly defined nuclear membrane. In *E. coli* the nucleus is almost invariably visible, is situated near the center of the organism, contains a great deal of chromatin, is large, and possesses a very thick, well-defined nuclear membrane.

*Vacuoles and contained bodies.*—In *E. histolytica* a vacuole is always present, except in the smallest individuals, and generally there is more than one. In *E. coli* a vacuole is generally absent, and more than one is of very rare occurrence. In *E. histolytica* the endoplasm is very often observed to contain one or several red blood corpuscles, while in *E. coli* red blood corpuscles are very, very rarely observed in the endoplasm, and experiments by adding blood to the feces containing

these amebae show that they do not engulf the red cells, while the same experiment demonstrates that many red blood cells are engulfed by *E. histolytica*.

*Motility*.—In *E. histolytica*, in freshly voided feces, the motility is marked, the organism progressing quite rapidly in a more or less definite direction. *E. coli*, under the same circumstances, possesses very sluggish motility, and often none at all, while it is almost never seen to progress in a definite direction. The motility alone will serve to distinguish these organisms, if both be observed in freshly voided feces, for it will invariably be found that the amebae exhibiting the most active form of motility will present morphological features which prove that it belongs to the pathogenic species.

To sum up: if, in a freshly voided specimen of feces we observe large, motile amebae, showing a clear, hyaline ectoplasm, the distinction between the ecto- and endoplasm being marked; an absence of a nucleus, or a nucleus situated near the periphery of the endoplasm, small in size, poor in chromatin, and having a dimly defined nuclear membrane, with two or more vacuoles, and with or without red blood corpuscles in the endoplasm, we may be sure that the organism observed is *E. histolytica*, for the amebae occurring in the feces of healthy individuals or in those of patients suffering from diseases other than amebic dysentery do not present the above characteristics.

*Stained specimens*.—In well-stained preparations the two species of ameba can be distinguished by the difference in the staining reactions of the ecto- and endoplasm. In *E. histolytica* the ectoplasm stains more intensely with Wright's stain than does the endoplasm, while in *E. coli* the opposite is true. This applies to specimens observed in the feces. In stained preparations showing reproductive forms *E. histolytica* can easily be distinguished by the occurrence of the various stages of sporulation described.

*Method of reproduction*.—To one who cares to devote the time and study necessary, the investigation of the methods of reproduction of the two species of amebae described will prove a certain method of differentiating them. Both *E. histolytica* and *E. coli* reproduce by simple division when conditions are favorable for a vegetative existence; when unfavorable conditions arise, *E. histolytica* reproduces by spore formation, a method totally different from that of *E. coli*, which,



under similar conditions, proceeds to encystment, and the formation within the cyst, of eight daughter-amebae. Both these methods have been described, but it should be insisted upon that this difference is all that is necessary to prove the existence of two species of amebae in the intestine of man.

#### CONCLUSIONS.

From my further study of this subject I see no reason for changing the conclusions arrived at in a former communication, and which are given briefly at the beginning of this paper. I believe that at least two species of amebae infest the intestine of man, one, pathogenic, the other, a harmless commensal. To the pathogenic species the name *Entamoeba histolytica*, given by Schaudinn, should be applied, while for the non-pathogenic species the name *Entamoeba coli*, also given by that investigator, must be retained. My belief regarding the existence of the two species of amebae is based upon the following facts:

First, the occurrence of amebae in approximately 50 per cent or more of healthy individuals and of individuals suffering from other diseases than dysentery.

Second, the occurrence of amebae in every case of amebic dysentery.

Third, the marked morphological differences between the amebae found in health and in diseases other than dysentery, and the amebae found in dysentery.

Fourth, the diversity in the method of reproduction of the amebae found in health and in diseases other than dysentery, and of the amebae found in dysentery.

Fifth, the production of typical amebic dysentery in kittens by the amebae present in dysentery, and the total absence of pathogenic action in kittens of the amebae occurring in health and in diseases other than dysentery.

But one conclusion is possible when the data given in this paper are considered: viz., that the intestine of man harbors at least two species of amebae, differing in their morphology, in their method of reproduction, and in their effect upon susceptible animals.

NOTE.—While correcting proof my attention has been called to an article by Walker, published in the *Jour. Med. Res.*, 1908, 17, p. 379, entitled "The Parasitic Amebae of the Intestinal Tract of Man and Other Animals," in which the author

from a study of cultures of various amebae, concludes that Schaudinn's classification is incorrect. Walker has fallen into the error already referred to, of comparing the morphology and reproduction of amebae in cultures with the morphology and methods of reproduction of amebae in the feces. While he calls attention to the erroneous conclusions which may be formed by a study of cultural forms, and states that control observations should be made of the organisms in their natural habitat, it does not appear that he has observed the development of *E. histolytica* in the feces in a single instance, his only material being a single culture of an ameba isolated from a dysenteric stool by Musgrave and Clegg. All of his other observations were made upon cultures of ameba from other animals than man, and from water and dead leaves. This author, while claiming that the morphological differences noted by Schaudinn between *E. Coli* and *E. histolytica* are trivial, does not hesitate to describe several new species which are based upon similar and, in some instances, even more trivial morphological differences. He was not able to cultivate ameba from the feces of six men, two of them suffering from amebic dysentery. His description of spore formation in the cultures of ameba studied is not inconsistent with the description given by Schaudinn, except that in *E. histolytica* the spores are liberated by a process of budding or gemmation. Walker states that Schaudinn's results have never been confirmed, despite the fact that my paper confirming them was published as long ago as 1905, and almost every systematic worker upon the subject has confirmed them since then. He states that he has not been successful in staining ameba with ordinary stains, but he was working with cultured organisms, and I have never been able to stain any ameba grown upon cultures with Wright's stain, which would appear to indicate that grave structural changes occur during cultivation. I believe that it is obvious that the author's conclusions regarding Schaudinn's work are unjustified, when it is remembered that he did not study *E. coli* at all, and that the only dysenteric ameba studied by him, so far as his paper indicates, was an organism sent him in culture by Musgrave and Clegg, and isolated by the latter authors from a dysenteric stool. It is evident that such an organism, cultivated for several months, and studied under cultural conditions, is an unsafe guide in the study of *E. histolytica*, as observed in the feces of dysenteric cases.

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## EXPLANATION OF PLATES.

## PLATE 2. FIGS. 1 TO 12. ENTAMOEBA COLI.

FIG. 1.—*Entamoeba coli*, showing nucleus with heavy nuclear membrane. No ectoplasm visible.

FIG. 2.—*Entamoeba coli*, showing a small vacuole, and large nucleus, with well-marked nuclear membrane.

FIGS. 3, 4, 5, and 6.—*Entamoeba coli*, motile form, showing well-marked nucleus and lack of distinction between the ectoplasm and endoplasm. The pseudopodia, composed of ectoplasm, resemble very closely in structure the endoplasm.

FIG. 8.—*Entamoeba coli* showing nucleus and crystals in protoplasm.

FIGS. 8, 9, 10, 11, and 12.—Stages in the reproduction by encystment of *Entamoeba coli*.

FIG. 8.—Primary division into two masses, containing protoplasm and nucleus.

FIG. 9.—Formation of double cyst-wall and division into active and passive pronuclei.

FIG. 10.—Fusion of the active and passive pronuclei within the cyst.

FIG. 11.—Division of the synkaryon, formed by the fusion of the active and passive pronuclei, into eight young amebae. Note character of cyst wall.

FIG. 12.—Thinning of cyst wall preparatory to liberation of the young amebae.

## FIGS. A TO H. ENTAMOEBA HISTOLYTICA.

FIG. A.—Diagram of *Entamoeba histolytica*. 1, Ectoplasm; 2, Endoplasm; 3, Vacuoles; 4, Crystals; 5, Red blood corpuscles; 6, Bacteria; 7, Nucleus.

FIG. B.—*Entamoeba histolytica*, showing two vacuoles and well-defined, hyaline ectoplasm. In this and the succeeding figures note the clear distinction between the ectoplasm and the endoplasm, as shown in the perfectly hyaline pseudopodia, which are composed entirely of ectoplasm.

FIG. C.—*Entamoeba histolytica*, showing small nucleus with thin membrane and two engulfed red blood corpuscles.

FIGS. D, E, and F.—*Entamoeba histolytica*, showing absence of nucleus, vacuoles, and red blood corpuscles in the endoplasm.

FIG. G.—*Entamoeba histolytica*, showing vacuolar degeneration.

FIG. H.—*Entamoeba histolytica*, showing vacuoles, red blood corpuscles, and nucleus.

## PLATE 3. FIGS. A TO H. ENTAMOEBA COLI, STAINED BY WRIGHT'S METHOD.

FIG. A.—*Entamoeba coli*, showing deeply stained protoplasm and nucleus.

FIGS. B and C.—*Entamoeba coli*, showing deeply stained endoplasm and more lightly stained ectoplasm. Also large nucleus, rich in chromatin.

FIG. D.—*Entamoeba coli*, showing staining reactions of ectoplasm, endoplasm, and nucleus. A small vacuole is present.

PLATE 2.



1



2.



3.



4.



5.



6.



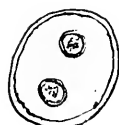
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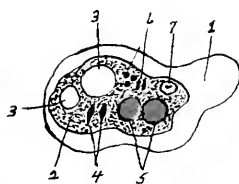
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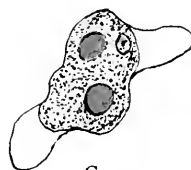
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A.



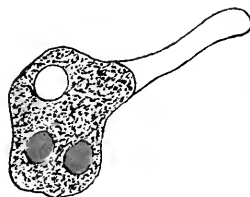
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C.



D.



E.



F.



G.



H.

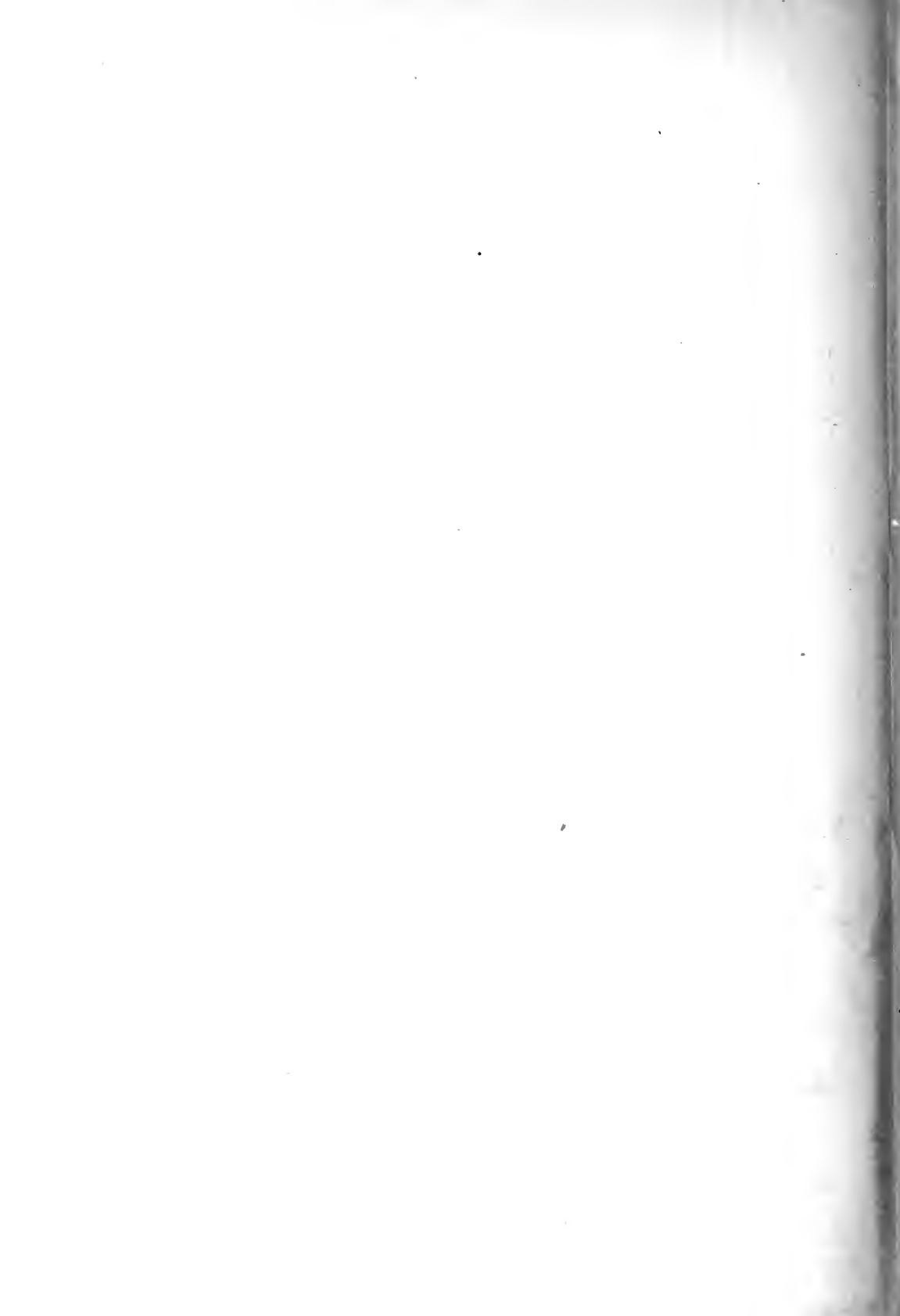
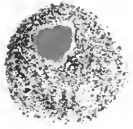


PLATE 3.



A.



B.



C.



D.



E.



F.



G.



H.



1.



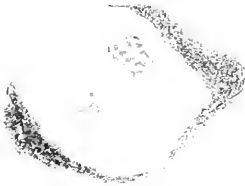
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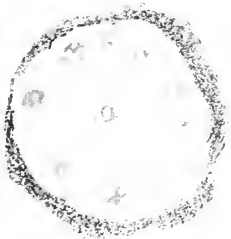
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6.



7.



8.



9.



10.





FIGS. E, F, G, and H.—Various stages in reproduction by simple division of *Entamoeba coli*.

FIGS. 1 TO 10. *ENTAMOEBA HISTOLYTICA* STAINED BY WRIGHT'S METHOD.

FIG. 1.—*Entamoeba histolytica* showing staining reactions of ectoplasm, endoplasm, and nucleus. Note the light staining of the endoplasm, the deep staining of the ectoplasm, and the smaller nucleus, less rich in chromatin, than the nucleus of *Entamoeba coli*.

FIG. 2.—*Entamoeba histolytica*, showing deeply stained pseudopodia, nucleus with some free chromatin, bacteria, and dimly stained endoplasm.

FIG. 3.—*Entamoeba histolytica*, showing absence of nucleus, characteristic staining of ectoplasm and endoplasm, and three red blood corpuscles.

FIG. 4.—*Entamoeba histolytica*, showing numerous vacuoles, and characteristic staining.

FIGS. 5, 6, 7, 8, and 9.—Stages in the reproduction of *Entamoeba histolytica* by spore formation.

FIG. 5.—Organism showing chromatin of nucleus arranged in rods and granules. The vacuole present contains some stained material.

FIG. 6.—Organism showing escape of the chromatin from the nucleus.

FIG. 7.—Organism showing distribution of the chromatin throughout the endoplasm, in the form of rods and granules.

FIG. 8.—Organism showing the collection of the chromatin into clumps about the periphery near the ectoplasm.

FIG. 9.—Organism showing the collection of the chromatin in the ectoplasm and the separation of the spores from the parent ameba by a process of budding.

FIG. 10.—Spores of *Entamoeba histolytica* as seen in the feces. The spores are bile stained and cannot be stained by Wright's method.

NOTE.—All of these drawings were made from specimens observed under the microscope using a 1-inch eye-piece and a  $\frac{1}{2}$ -inch objective.



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## THE RATE OF MULTIPLICATION OF *BACILLUS COLI* AT DIFFERENT TEMPERATURES.\*

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IN estimating the rate of reproduction of bacteria, the following quantities must be known: the number of bacteria at the beginning of a definite period of growth, the number at the end, and the length of time over which the experiment extends. If the number at the start be represented by  $a$ , the number at the end by  $b$ , and the number of generations by  $n$ , we have the equation:

$$2^n = \frac{b}{a}.$$

Then if  $G$  be the time required to complete a full generation,

$$G = \frac{t}{n}.$$

where  $t$  is the time covered by the experiment. The validity of the equation  $2^n = \frac{b}{a}$  depends, of course, on the assumption that each bacillus divides into two nearly equal parts, and that all of the bacteria in a culture reproduce at nearly the same rate. In order that

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this even rate be kept up, conditions must remain constant during the experiment; and in order that the maximum rate for a given temperature be maintained, the following additional requirements, included in the list given by Buchner, Longard, and Riedlin, must be observed: A favorable liquid culture medium and a pure and actively growing culture must be used. The initial number of bacteria must be small, and the time of the experiment must not be long enough to allow an accumulation of products of metabolism sufficient to inhibit growth.

The estimation of the generation time of different species of bacteria has been attempted by various authors and by different methods. Nägeli and Schwendener<sup>1</sup> sought to use the amount of acid formed by certain fermenting bacteria as a criterion of numbers. Buchner, Longard, and Riedlin,<sup>2</sup> Rahn,<sup>3</sup> and Müller<sup>4</sup> used plate cultures for the determination of  $a$  and  $b$  in the above equation. Boland<sup>5</sup> used a turbidity standard in the estimation of numbers, and compared cultures with standard turbidity solutions made by talc suspensions. Klein<sup>6</sup> and Hehewerth<sup>7</sup> determined  $a$  and  $b$  by the direct counting of cultures stained in the moist condition.

My own work on this problem was attempted in the hope of obtaining more accurate results by the use of a new technique which I have developed in connection with other research.<sup>8</sup> Some modifications of this technique, developed in connection with the work here described, are as follows:

A large sterile cover is placed over the moist box ( $b$ , Fig. 1) and a drop of the culture to be used is placed under this cover. Drying is prevented by the saturated filter paper lining the sides of the moist box and by an abundance of water placed in the bottom. A moist atmosphere is further insured by placing numerous drops of sterile broth, gelatine, or agar on the under surface of the cover around the field of operation. The sterile pipette,  $p$ , the inner end of which is drawn out into a very fine capillary tip, is partly filled with the broth to be used and adjusted in the holder  $kg$ , in such a position that the tip may be brought into focus in the center of the field

<sup>1</sup> Das Microscop., 2. Aufl., Leipzig, 1877, p. 641.

<sup>2</sup> *Centralbl. f. Bakt.*, 1887, 2, p. 1.

<sup>3</sup> *Ibid.*, 1906, Abt. 2, 16, p. 417.

<sup>4</sup> *Archiv f. Hyg.*, 1903, 47, p. 127; *Ztschr. f. Hyg.*, 1895, 20, p. 245.

<sup>5</sup> Inaug. Dissert., Amsterdam, 1902.

<sup>6</sup> *Centralbl. f. Bakt.*, 1900, 27, p. 834.

<sup>7</sup> *Archiv f. Hyg.*, 1901, 39, p. 321.

<sup>8</sup> "On Heredity in Certain Micro-organisms," *Kansas University Sci. Bull.*, 1907, 4, No. 50, p. 3.

of the objective to be used. The culture drop containing the bacteria is brought into the field by means of the mechanical stage, and the pipette raised by screw *f* until its tip is in contact with the culture. One or more bacteria will enter the pipette by force of capillarity. The pipette is then quickly lowered, and, by moving the mechani-

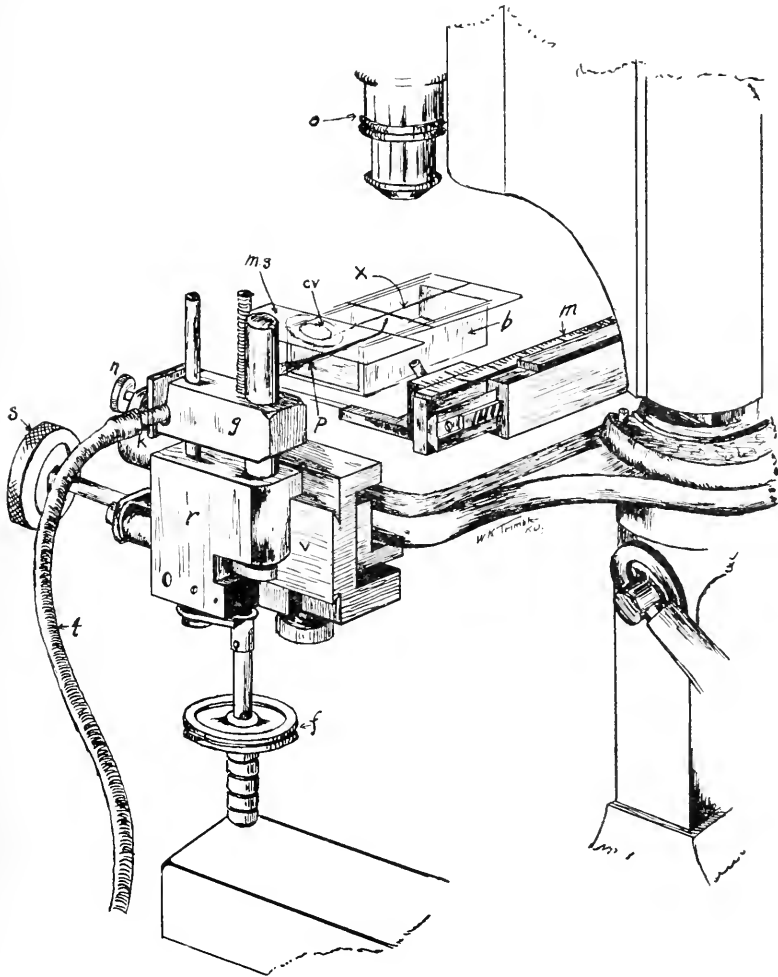


FIG. 1.

cal stage, a portion of the cover remote from the culture drop is brought into view. The pipette is then raised, and, by gently blowing into the rubber tube *t*, a portion of the contents containing the bacteria is discharged on the cover. If the bacteria are not too thick in the drop from which selection is made, and if the aperture of the pipette is sufficiently small, a single bacillus may be drawn into the pipette. If several are

taken up, it is easy to separate them by subsequent dilutions on the cover. In preparing for a hanging drop series I have usually isolated several individuals, so that a selection can be made from the most actively growing culture at the second isolating. The further procedure is described below.

I have used in some of these experiments a modified pipette holder constructed to hold two pipettes. In this holder each pipette may be raised or lowered independently of the other, and they may be so adjusted that both capillary tips may be brought into the same field. An additional adjustment, provided with screws and arranged in block *g* (Fig. 1), allows these pipettes to be moved independently in and out, as well as up and down. This apparatus dispenses with the necessity of changing pipettes between the first and second isolations. However, in view of the ease with which a new pipette may be made and inserted, I have preferred for most experiments the simpler type of holder shown in the illustration.

When a bacillus is to be transferred to a test-tube, it is usually necessary to isolate the bacillus with one pipette and to transfer it to the test-tube with a fresh sterile one, so that one may be sure that there is but one organism transferred. A new pipette then must be made for each bacillus. In order to simplify this process and to use the same pipette for several transfers, the following modification of the process has been devised: A piece of mica, about  $2 \times 4$  cm. in size, is cut out and in the center a round aperture about 8 mm. in diameter is made. Over this aperture a clean cover-glass, about  $\frac{1}{2}$  in. in diameter, is placed and the cover, together with the mica holder, is sterilized in the Bunsen flame and placed over the moist box in the position shown in the illustration. (*ms* and *cr*, Fig. 1.) The mica slip, shown at the left side in the illustration, may be placed at the opposite end of the moist box. In either case the large cover, *x*, is slipped to one side or the other to accommodate it. A drop of sterile broth is placed under the smaller cover either after it is placed on the moist box or just before. Then by means of the pipette, already in position, a single bacillus is taken up from the droplet in which it had previously been isolated. The cover on the mica slip is then brought into the field by means of the mechanical stage, the pipette is raised, and the bacillus deposited in the drop of broth under the smaller cover. An ordinary platinum loop, somewhat flattened, is sterilized and dipped into sterile broth. The wetted loop is brought into contact with the top of the small cover, and the cover lifted and deposited, together with the hanging drop and single bacillus, in a tube of sterile broth. The mica may now be sterilized anew, again put into place with a new cover and a second bacillus isolated. The bacillus may be seen moving about in the droplet under the smaller cover, so that one is sure that it is in an active condition and is transferred in this state to the test-tube.

This method may be used either with or without the warm box described below. A bacillus may be isolated, and left over the moist box protected by the warm box until two generations have been formed. Then each of the four bacilli may be taken out and deposited in four separate test-tubes, using the same pipette for each. Contamination may be avoided by arranging a hood of moist cloth or paper over the portion of the microscope used in the process. In the course of many isolations by this method, I have thus far had no contaminations. The method is a convenient one for isolating single organisms from mixed cultures. The single organism may be transferred to liquid or solid media.

The warm box used is made on the plan of the Zeiss warm box with metal base, glass front, and hinged, wooden sides. Heat, in my apparatus, is maintained by

resistance coils placed under the base of the microscope, and the temperature is kept constant by an electro-thermoregulator arranged to automatically connect or disconnect the heating current. I have protected the top and sides of the box with a jacket made of two layers of felt with asbestos wool between the layers. In this apparatus a very constant temperature may be maintained.

In preparing for an experiment the microscope is placed in the box, the light properly focused, and the pipette adjusted in position under the cover-glass holding the hanging drops. Of the microscope only the tube and the fine and coarse adjustments project from the closed box. Apertures are provided for the rubber tube, *t*, and for rods by which the mechanical stage is manipulated. A small door is placed in the left side of the box in order that the thumb may be inserted for manipulating screw *f* of the pipette holder. So a series, involving many isolations of bacteria, may be continued for hours without opening the box or altering the temperature.

There is a tendency, in the warm box, for moisture to collect on the part of the large cover which lies immediately over the center of the stage, since the heat, coming from below, passes around the condenser and causes the moisture in the bottom of the glass box to form a slight vapor. So, by moving the portion of the large cover holding the series of hanging drops to the right or left of the center of the stage immediately after an observation, the amount of moisture on that part of the cover may be nicely governed.

In order to carry on this work successfully, it is necessary to work with a strong light, especially when the warm box is used. For this I employ a Welsbach burner; and, in order to partially free this light from yellow rays and to focus it strongly on the mirror, I use a large spherical glass globe condenser filled with a weak solution of copper sulphate.

Much depends on using pipettes with a very small aperture at the capillary end. I have somewhat improved the method of making these pipettes and proceed as follows: One end of a thin-walled glass tube, 8 to 10 cm. in length and about 4 mm. in diameter, is drawn out in a small flame into a thin-walled capillary tube about 0.5 mm. in diameter. The capillary end is again drawn out over a very small, narrow flame produced by a micro-burner. This small flame should be only 3 or 4 mm. in height. If the capillary tube is held at a proper distance above this flame and drawn out with some force the instant the glass softens, apertures of very narrow diameter and with smooth edges may be made. About 5 mm. of the tip is then bent at right angles in the flame and the pipette is ready to be filled with the medium. This may be taken directly from a test-tube. If, on inserting the tip into the liquid, it is found that there is no aperture present, one of sufficient fineness may often be made by scratching the tip *very gently* on the sides of the test-tube. The size of the aperture may be gauged approximately by the rapidity with which the liquid enters the pipette when suction is applied through the rubber tube. One may make and successfully use pipettes having an aperture not exceeding a small yeast cell in diameter. Much coarser ones may be successfully used, but where the aperture is too large the labor is much increased and the accuracy of the work diminished.

The essential feature of the method I have employed is that each experiment is begun with a single bacterium. Thus *a* of the equation given above is reduced to unity, the equation simplified to  $2^n = b$ ,

and the errors incident on the determination of  $a$  are reduced to the minimum. Further, on account of the small magnitude of  $a$ , a considerable time may be employed and a considerable number of generations formed while  $b$  is still relatively a small number. This facilitates the determination of  $b$ ; and, in making  $n$  relatively large, reduces the margin of error. Moreover, the relatively small number of bacteria in the culture minimizes the danger of inhibition of growth due to the accumulation of products of metabolism. Again, when a considerable number of bacteria are used as a starter, there are likely to be some less vigorous ones included, however active the culture from which the starter is taken. By selecting as a starter a single healthy bacterium from the most vigorous individuals of a culture, one may avoid this error and begin the experiment under optimum conditions. The term "single bacterium" is made to include some cases in which two or more elements were joined in a short chain.

One race of *B. coli* was used during the whole work, a strain obtained from the collections of the Pasteur Institute in 1904. During the two years or more during which these experiments were conducted the growth rate of this strain of *B. coli* was frequently tested under similar conditions of medium and temperature, and there was no evidence of degeneration or other change. In practically all experiments, beef peptone broth was used.

Three variations in the above method were used in ascertaining the growth rate. The bacilli were cultivated in hanging drops over hollow slides, in a series of hanging drops kept in a warm box, and in test-tubes. In the warm-box and test-tube series only a limited number of temperatures was employed, since these methods were used largely to confirm the results obtained by the hanging-drop method.

In the hanging-drop method the following procedure was followed in most cases. A single bacillus was isolated and allowed to stand in the medium and at approximately the temperature at which it was subsequently to be grown, until one, two, or sometimes three generations had formed. Then with a fresh pipette, the two, four, or eight similar units were distributed each into a separate hanging drop, and one of these units killed and stained to serve as a control of the size of the others. The cover was then sealed over a hollow



slide, placed at the appropriate temperature, and left a definite period of time. Temperatures above room temperatures were, in a majority of cases, maintained in an incubator or water bath heated by electric heat governed by an electrothermostat; so that these temperatures were in most cases very constant. Below room temperature, various devices, usually depending on cold water, either flowing from the tap or otherwise arranged, were employed. In a few cases, a refrigerator was used, but this was not found to be sufficiently reliable.

At the close of an experiment the cover-glass was again placed over the moist box and the cultures were killed and stained by adding to each hanging drop a small quantity of a reagent composed of a saturated solution of methylene blue to which was added, just before use, a small quantity of a 5 per cent solution of potassium hydrate. The addition of this reagent was made under the microscope and with the aid of the pipette previously used in isolating, or of a similar pipette, so that a small enough quantity could be used to instantly kill and stain without adding enough color to the drop to interfere with the counting. The counting was done in the hanging drop and the comparison made with the control in order to estimate generations in units of the same size as those used in the starters. If the generations did not come out even in a given series, the time was usually varied in the next experiment so as to make the number of generations come out approximately even. In some instances no control was kept, and the size of the starter estimated by direct measurement with the eyepiece micrometer. Tests were usually confirmed by experiments made at the same time and temperature and with the same medium. In many cases, further confirmatory tests were made at the same or approximate temperatures and with a different lot of medium.

The results of these experiments are given in Table 1. In some cases the values in the *n*-column are not whole numbers. Here the decimal was reckoned on the basis of the size of the units at the end of the series as compared with the control. In other cases two even values of *n* are given for the same experiment, values between which the true value lies. Such numbers are inclosed in brackets in which are also given the appropriate values of *G*.

The numbers in the fifth column indicate the number of hanging drops which gave the values which precede. If the number 2 is given it is meant that the corresponding experiment was done in duplicate; if 3, in triplicate, and so on. The numbers marked with asterisk in the *G*-column are those considered to be the minimum reliable values, and it is largely on the basis of these marked numbers that the curve given below is plotted. These values are selected because determined by experiments conducted under optimum conditions and apparently relatively free from error. Further, these selected values were in most cases confirmed by a considerable number of duplicate experiments. Since the intention of the experiments is to obtain the maximum rate rather than the average rate, these selected values are probably more reliable than averages, though the averages at most of the temperatures closely approximate the values selected.

Practically all the determinations made during the work are given in the table, the ones which vary greatly from the minimum as well as the ones which closely approximate it, since it is desired to show the amount of variation in the results. Only certain determinations made while the method was being developed, or results based on experiments known to be faulty, are omitted.

It is shown in Table 1 that the growth of this race of *B. coli* begins at about 10° and the rate rises rapidly to about 37°5 where it reaches a minimum generation time of nearly 17 minutes. It remains at a nearly constant high rate until about 45° when it falls rapidly and nearly ceases at 48°. The temperature curve is shown much more graphically in the chart given below and further discussion of it is deferred to that part of the paper.

The values obtained for *G* in the lower temperatures vary greatly. This is due in part to the degeneration often noted in the cells at very low temperatures. It is also partly due to the difficulties of keeping constant low temperatures for the long time necessary to obtain a considerable number of generations. For when only two or three generations occur in a relatively long space of time, the margin of error is great. The irregularities observed in the rate of reproduction in the higher temperatures were correlated with degeneration of cells, tendencies to grow into long threads, and

TABLE 1.  
MULTIPLICATION OF *B. Coli*. HANGING-DROP METHOD.

Tempera- ture	Time in Minutes	No. of Gen.	Genera- tion Time in Minutes (G)	No. of Deter.	Tempera- ture	Time in Minutes	No. of Gen.	Genera- tion Time in Minutes (G)	No. of Deter.
	(t)	(n)				(t)	(n)		
5.5-6.2			Growth		30.0	208	7	29.7*	4
5.8-6.4	7 hrs.		none or		30.2	180	6	30	2
5.9-6.4	5½ hrs.		very		30.5	165	6	27.5	2
7.0-8.5	5 days		slight		31.6	240	8	30.0	1
9.0-11.0	25 hrs.	2		4	31.6	253	8	26.7	1
9.5-10.5	1044	2	750	2	31.6	420	17	31.6	1
10.0	2595	3	865*	2	31.6	202	8	24.7	1
12.5	1140	2	570*	2	31.6	196	8	26.3	1
12.6-13.9	360	2.2	164		31.6	175	7	25.1*	1
13.6-14.3	755	3.5	214*	2	32.0	150	6	24.5	1
13.3-13.8	586	3.5	182	1	33.6	257	10	25.0	5
14.2	1285	6.5	198*	1	33.6	250	11	25.7	4
14.3	1168	6.5	194	4	33.6	185	8	23.4	1
15.4-15.8	805	101	180	5	33.6	125	5.5	25.0	1
16.0-16.8	1570	13	120.8*	2	33.6	128	5.5	23.7	4
18.1	470	5	94	6	33.6	115	5.0	25	6
18.5-18.8	565	5.2	108	2	33.6	132	5	23.3	3
18.5	490	5	98*	4	33.6	120	5	25.0*	2
19.5	390	4	97.5	1	33.6	71	3	26.4	2
19.5	558	5.3	105.3	4	33.6	77	3	22.0	3
19.5	765	8	95.6*	1	33.6	240	11	21.8	2
21.5-21.8	355	5.7	62.2	3	33.6	141	6	23.5	2
21.6	420	7	60*	5	33.6	132	6	22.0	1
22.6-23.0	318	6	53*	3	33.6	226	10	22.6	2
23.6	226	5	45.2	2	34.0	120	5.3	21.8	2
23.6	407	8	50.9*	4	34.4-35.6	160	7	22.9	1
23.6	454	9	50.4	2	35.0	145	6.5	22.3	1
23.6	348	7	49.7	1	35.0	170	7	24.3	1
23.6	495	8	50.8	1	35.0	136	6	22.6	2
23.6	360	7	51.4	1	35.0	120	5.5	22.0	2
23.7-24.1	276	5	54.6	3	35.0	132	6	22.0*	2
24.1	240	4.8	50*	1	35.0	187	8	23.4	1
24.6-25.5	164	4	41*	4	35.0	187	7	25.5	1
25.2-26.0	205	5	41	2	35.0	145	8.5	22.0	1
25.5	362	9	40.2	1	35.0	120	5	23.0	1
25.5	443	11	40.3*	2	35.0	117	4.8	24.0	1
25.5	303	8	37.0	1	35.0	134	6	22.3	1
25.5	333	8	41.6	1	35.0	130	6	21.8	1
25.5	423	10	42.3	1	35.1-35.2	148	6.5	22.8	1
25.5	410	10	41.1	2	35.1	132	6	22	4
27.6	183	5	36.5	1	35.1	120	5.5	20.3	4
27.6	238	6	39.7	1	36.0	120	6	20.0*	4
27.6	212	6	35.3	1	37.0	120	6	20.0	6
27.6	230	7	38.3	1	37.0-37.4	120	6.2	10.4*	2
27.6	395	11	35.9*	1	37.2-37.4	120	6.5	18.5	4
27.6	494	14	35.3	1	37.5	111	5.5	20.2	1
27.6	404	11	30.7	1	37.5	120	7	17.2*	7
27.6	251	7	35.0	1	37.5	185	9	20.6	1
27.9-28.0	185	6	30.8	4	37.7	131	7	18.7	2
29.5	180	6.5	27.7	3	37.7	121	6	20.2	5
29.6	156	5	30	3	37.7	233	11	21.2	1
29.6	163	5	31.2*	1	37.7	116	6	19.3	2
29.6	163	5	32.6	4	37.7	106	5	21.2	2
29.6	376	13	28.9	1	37.7	155	8	19.4	2
29.6	180	13.5	27.9	1	37.7	101	5	20.2	1
29.6	162	6	26.1	3	37.7	100	5	20.0	8
29.6	311	11	30	3	37.7	99	5	19.8	2
29.6	493	15	32.4	2	37.7	120	6	20.0	6
29.6	420	13	28.3	1					
29.6	311	10	38.2	2					
			32.2	1					
			31.1	2					

TABLE 1.—Continued.

Temperature	Time in Minutes	No. of Gen.	Generation Time in Minutes (G)	No. of Deter.	Temperature	Time in Minutes	No. of Gen.	Generation Time in Minutes (G)	No. of Deter.
37.7	98	5	19.6	1	44.5	150	6	25	3
38.1-39.2	127	6	21.2	1	44.5	138	7	19.7*	3
38.2	120	6	20	1	44.6	146	7	20.0	3
38.5	120	7	17.2*	11	44.6	169	7	24.1	1
38.5	120	6	20.0	5	44.6	122	6	20.3	4
38.5	117	6	19.5	2	44.6-44.8	110	5	22.0	5
38.5	119	6	19.8	3	44.6-44.8	125	6	20.8	5
38.5	132	7	18.4	1	44.8	125	6	20.8	1
38.5	139	7	17.4	1	44.8	125	7	17.9	1
38.5	119	7	18.9	1	44.8	220	12	18.3	1
38.5	174	9	17.0	1	44.8	220	11	20.0*	2
38.5	174	9	10.3	1	44.8	159	7	22.7	2
38.5	154	9	17.1	1	44.8	127	6	21.2	1
38.5	172	9	10.1	1	44.8	127	7	18.1	1
38.5	171	10	17.1	1	44.8	136	6	22.7	2
38.5	102	5	20.4	1	44.8	102	5	20.4	1
38.5	130	7	18.6	2	45.0	129	6	21.5	1
38.5	125	7	17.9	1	45.2	99	4	24.8	1
38.5	183	10	18.3	1	45.2	120	4	30.0	1
38.5	123	7	17.6	3	45.2	120	5	24.0	1
38.5	158	9	17.6	1	45.2	120	6	20.0*	3
39.6	119	7	17.0	1	45.2	268	9	29.8	1
39.6	121	7	17.3	2	45.2	91	4	22.8	2
39.8-40.2	120	7	17.2	3	45.2	120	4	30	1
40.7	180	5	36.0	1	45.6	137	6	22.8	3
40.5	120	7	30.0	1	45.6	114	4	28.5	3
40.8	150	6	17.2*	5	45.8	102	5	25.0	1
40.8	150	7	28.3	4	45.8	102	3	34.0	2
40.8	120	7	24.3	1	45.9-46.1	93	3	31.0	1
40.8	190	10	17.2	1	45.9-46.1	171	7	24.4	3
40.8	124	7	10.0	1	45.9-46.1	171	6	28.5	2
40.8	120	7	17.7	3	46.0-46.2	120	2	60.0	1
42.8	118	6	17.2	4	46.3-46.5	142	5	28.4	3
42.8	120	6	19.7	2	46.5	165	6	27.5	1
42.8	120	6	20.0	3	46.5	152	3	50.6	2
42.8	156	8	19.5	1	46.8	137	2	68.5	1
42.8	156	7	22.3	2	46.8	137	3	45.6	1
43.3-43.8	120	6.8	17.7*	2	46.8	183	4	45.7	1
44.2	128	6	21.3	2	46.8	180	4	45.0	1
44.2	116	7	18.3	1	46.8	457	10	45.7	3
44.2	122	5.5	23.5	1	46.8	457	11	41.5	2
44.3	124	6	22.1	4	46.9-47.3	195	1	2 generations	1
44.5	125	5	20.3	3	47.0-48.5	Growth No	slight and irregular growth observed	ar	
			25.0	2					

other morphological irregularities. These with the failure of some of the cells to grow make determinations in the region above 45° comparatively unreliable.

In the second method the warm box described on p. 382 was used. Here the cover-glass holding the isolated bacilli was not removed from the moist box (see *b*, Fig. 1) on the stage of the microscope, and the experiment was kept under observation during the whole time of a series, sometimes extending over a space of 12 hours. A single bacillus, well accustomed to the temperature and medium and vigorously growing, was isolated and allowed to grow to usually two and sometimes three generations. Then one of the daughter

cells was removed by the pipette and placed alone in a new drop. When this had attained two or three generations one of its descendants was isolated, and so on. Thus, long series were carried out, the whole series kept in the warm box under nearly constant temperature and conditions of moisture, and exposed to the light only during the brief time required to make the isolations—often less than two minutes. At each fresh isolation, the time, temperature, approximate size of the unit transferred, and its motility were noted; so at the close of a long series not only the value given by dividing the whole time by the whole number of generations could be obtained, but values for lesser periods when the rate of reproduction may have varied. In a majority of the series the rate was fairly constant

TABLE 2.  
MULTIPLICATION OF *B. Coli.* WARM-BOX METHOD.

Temperature	Time Whole Series ( <i>t</i> )	Number of Generations Whole Series ( <i>n</i> )	Generation Time Whole Series ( <i>G</i> )	Time Partial Series ( <i>t</i> <sub>1</sub> )	Number of Generations Partial Series ( <i>n</i> <sub>1</sub> )	Generation Time Partial Series ( <i>G</i> <sub>1</sub> )
29.5-30.8.....	320	9	35.6	{ 184 123	6 4	30.7 30.8
29.8-30.4.....	335	11	30.5			
30.0-30.4.....	366	11	33.3			
33.0-33.8.....	736	30	24.5	{ 223 455	10 19	22.3 23.9
33.6-34.2.....	360	16	22.5			
35.8-37.4.....	525	26	20.2			
36.2-37.0.....	389	20	19.5	{ 303 236	16 12	18.9 19.6
36.6.....	205	9	22.8	148	7	21.1
37.1.....	345	15	23.0	152	{ 7 8	21.8 19.9
37.1-38.2.....	491	22	22.3			
37.1-39.6.....	543	30	18.1	{ 201 342 141	11 19 8	18.3 18.0 17.6
37.4-38.4.....	212	11	19.3	{ 327 140	18 8	18.2 17.5
37.4-38.1.....	313	17	18.4	{ 219 179 132	12 10 7	18.3 17.9 18.9
37.6-38.6.....	427	20	21.4	{ 427 125	20 6	21.4 20.8
37.6-38.4.....	303	16	19.0	240	13	18.5
37.6-37.9.....	414	20	20.7			
37.9-38.1.....	396	18	22.0	{ 146 118	7 6	20.9 19.6
38.1-39.1.....	254	15	16.9	{ 214 131	12 8	17.8 16.4
38.1-38.3.....	240	12	20.0	{ 172 82	10 5	17.2 16.4
38.3.....	720	38	18.9	213	13	16.4
38.3.....	201	10	20.1			
39.3-41.4.....	750	38	19.7	{ 550 459	29 24	19.0 19.1
40.3-40.9.....	486	24	20.3	126	7	18.0
43.8.....	240	10	24.0	182	8	22.5

throughout the whole time; but in some, on account of temporary lack of moisture or some other unfavorable condition, the rate sometimes fell temporarily below the maximum. In these series I have given in Table 2 not only the value for the whole series, but also values for partial series during which conditions were more favorable. I have also (for the sake of confirming the results of the whole series) included some partial series taken from experiments in which the rate was nearly constant. In all series, whether whole or partial, it was necessary, in fixing the end points, to take bacilli of approximately the same size.

It is seen on comparing results that the values obtained by this method confirm those of the hanging-drop series. The uniformity of rate of division in the longer series is worthy of note. Up to the thirty-eighth generation at least, there is no period of rest or slackening of the growth rate, and it is probable that this rate would continue indefinitely so long as conditions were kept constantly favorable.

In order to eliminate possible error due to varying concentration of the medium in the hanging drops, the isolated bacterium was, in some series, drawn into the pipette and allowed to divide there into two or four. All were then discharged on the cover and one alone of the number drawn into the pipette for further growth, and so on. These series gave approximately the same results as when division was allowed to proceed on the cover-glass.

In the two methods described above, growth took place in hanging drops. In a third method, the single bacillus, vigorously dividing and accustomed to the medium, was transferred directly to a test-tube containing usually 5 or 10 c.c. of sterile broth. This tube was then incubated at the desired temperature until the first cloudiness appeared, which was usually after eight to nine hours at the optimum temperature. The culture was then killed by the addition of two or three drops of formalin, and the bacteria counted unstained in the ordinary Zeiss or Leitz counting chamber. The size of a bacillus used as a starter was noted and this size taken into account in the final estimation of generations.

The number of bacteria, *b*, given in the third column (Table 3) includes the total number formed in the test-tube, and varies with the quantity of broth as well as with the cloudiness of the culture. In the

fourth column under  $n_1$  and  $n_2$  are given the even powers of 2 lying above and below the corresponding  $b$ , and under  $G_1$  and  $G_2$  are given the generation times corresponding to  $n_1$  and  $n_2$ . But one of these values is given where  $b$  closely approximates an even power of 2. In the sixth and seventh columns are found the values of  $n$  and  $G$  worked out according to the formulas given at the top of the respective columns.

TABLE 3.  
MULTIPLICATION OF *B. coli* IN TEST-TUBE CULTURE.

Temperature	Time in Minutes ( <i>t</i> )	Number of Bacteria Millions ( <i>b</i> )	Number of Generations ( $n_1$ ) ( $n_2$ )	Generation Time in Minutes ( $G_1$ ) ( $G_2$ )	$2^n=b$ ( <i>n</i> )	$G=t/n$ ( <i>G</i> )
30.0.....	775	94.2	27 26	28.7 29.8	26.48	29.3
	762	81.8	27 26	28.2 29.3	26.28	29.0
	770	70.0	27 26	28.9 29.6	26.06	29.5
	820	60.0	26 25	31.5 32.8	25.84	31.7
	835	57.8	26 25	32.1 33.4	25.78	32.4
35.0.....	573	126.5	27 26	21.2 22.0	26.71	21.3
37.0.....	600	1240.0	31 30	19.7 20.3	30.28	20.1
	538	111.75	27 26	19.0 20.7	26.74	20.1
36.5-37.5.....	522	98.41	27 26	19.3 20.1	26.55	19.7
	454	15.62	24	18.3	23.00	19.0
	465	30.40	25	18.6	24.86	18.0
	504	102.88	27 26	18.7 19.4	26.28	19.2
	484	38.46	25	19.4	25.10	19.2
37.0-37.3.....	532	144.7	28 27	19.0 19.7	27.18	19.0
	546	164.5	28 27	19.5 20.2	27.29	20.1
	511	103.5	27 26	18.0 19.7	26.62	19.2
	506	128.0	27 26	18.7 19.5	26.93	18.8
	561	160.67	28 27	20.0 20.8	27.34	20.5
37.2-37.5.....	478	135.0	27	17.7	27.09	17.7
	515	102.14	27 26	19.1 19.8	26.61	19.4
	534	324.0	29 28	18.4 19.1	28.28	18.0
	514	70.14	27 26	19.0 19.8	26.24	19.6
	483	30.12	25	19.3	23.84	19.4
37.2.....	491	51.45	26 25	18.9 19.7	25.63	19.2
	535	60.46	26	20.6	25.52	21.0
	569	156.0	28 27	20.3 21.1	27.22	20.0
37.3.....	549	333.0	29 28	18.0 19.8	28.31	19.2
37.5.....	507	108.0	27 26	18.8 19.5	26.60	18.3
	535	69.5	27 26	19.8 20.6	26.05	20.5
	549	102.0	27 26	20.3 21.1	26.61	20.6
	538	97.0	27 26	19.0 20.7	26.53	20.3
	553	66.0	26	21.3	25.97	21.3

It is seen that the results obtained in the test-tube cultures closely approximate those obtained by the other methods. It is evident by comparing  $G_1$  and  $G_2$  that the probable error in estimating numbers is small; for an error of 100 per cent in counting will amount to an error of only one generation, about 5 per cent in the value of  $G$ , and the average error in counting is probably under 10 per cent.

A certain proportion of the test-tube cultures inoculated with single bacilli failed to grow. This is more probably due to some error in transferring than to any effect of a large quantity of sterile

medium on a single bacillus. In the last series conducted with the most improved technic, all of a series of six bacilli grew when transferred each to a tube containing 5 to 10 c.c. of broth. A number of earlier failures were probably due to allowing the media surrounding the isolated bacillus to concentrate by partial drying before the bacillus was transferred. The newly formed bacilli seem especially sensitive to drying or excessive concentration of the medium.

Each of the three methods tends to eliminate the sources of error of the other two. The chief source of error of the first method, the smallness of the number of generations counted, is avoided in the second and third methods where the generation number is large. Inhibition of the rate of growth due to crowding is avoided in a warm-box method where usually no more than four bacilli are found in a drop at one time; and the agreement of the results obtained by this method with those of the other two methods makes it seem unlikely that either in the hanging drops or test-tubes a sufficient number of bacteria were allowed to form to inhibit growth by crowding. The estimation of the number of bacteria at the close of the experiment, while approximately exact in the test-tube experiments, can be made very exact in the hanging drops; and error from this source is avoided altogether in the warm-box method.

In the warm-box experiments where the one bacillus that is to serve as a starter for the next drop is chosen from two, four, or eight, there is a possibility of error in unconscious selection of larger or smaller elements, since division does not always result in the formation of exactly equal parts. This error has been avoided largely by selection of units of average size. This has been facilitated by isolating in some cases more than one of the units of a given drop and comparing the results of growth two or three generations later, thus following out the series in duplicate or triplicate. However, to ascertain the amount of error possibly due to unconscious selection, two series were carried out at the same time and under the same conditions, both series starting from a common ancestor. In one of these the largest of the units was selected at each transfer, in the other, the smallest. At the end of the entire time of the experiment, 476 minutes, the series in which the larger elements were selected gave 26 generations with a generation time of 18.3 minutes.



The series in which the smaller elements were chosen gave 23 generations, with a generation time of 20.7 minutes. So from this experiment in which the difference is less than 2.5 minutes, it may be concluded that the average error is not large when pains are taken to avoid selection.

Rahn, Hehewerth, and Müller<sup>1</sup> mention a phenomenon said to occur in their experiments which, if present here, would be an additional source of error. This is an inhibition, or even temporary stoppage, of growth occurring during the time immediately following the inoculation into the fresh broth of the bacteria used as a starter. The growth rate, at first very slow, is said to reach a maximum only after some time, and this maximum rate can continue only a comparatively short time until growth is checked by the accumulation of products of metabolism. This initial slow growth is the more marked the lower the temperature, and is less conspicuous when fresher cultures are used as starters and a larger number of bacteria sown. Rahn explains the more rapid growth following the initial pause by the production of a specific substance, comparable with the "bios" of Wildier, a substance that is produced by the bacteria and is necessary for their most vigorous growth.

In my experiments with *B. coli* I found absolutely no sign of this initial slow growth when rapidly dividing elements, accustomed to the medium, were used at the start. Several experiments were carried out in the warm box which clearly showed the absence of the initial slow growth. In these series the daughter cells were transferred to a new drop of fresh medium at the completion of the first division. Each hanging drop is then one generation older than the preceding, and the drops are made large enough to accommodate six or seven generations without crowding. After about two hours at the optimum temperature all the bacteria in the hanging drops of a series thus made are killed and stained. The resulting numbers in a series are shown in the following diagram:

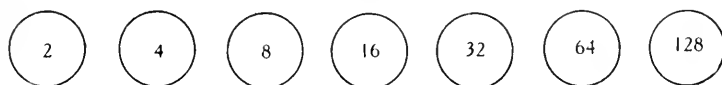


FIG. 2.

<sup>1</sup> *Loc. cit.*

If two generations are allowed to form in any one drop and three instead of one left behind at transferring, the three continued to divide at the same rate as the others but according to the series: 3, 6, 12, 24, 48, 96, etc. If there is an increased rate of reproduction after a considerable number of bacteria are formed we would expect a larger number than 64 or 128 in the older drops. In large hanging drops this series may be continued one or two generations further without evidence of any increasing rate of reproduction.

Further, if a certain concentration of bacteria is necessary to rapid growth, one would expect a very slow growth for some hours following the introduction of a single bacillus into 10 c.c. of broth. That this is probably not the case is shown by the fact that the generation time of such cultures for a long series is approximately the same as that in hanging drops where a considerable concentration of bacteria is soon reached. Further, in the warm-box experiments the rate is as rapid as in the hanging drop or test-tube, though in this case fresh broth is added with each transfer and the transfer may be made before more than two bacteria are formed in a drop. These experiments certainly argue against the theory that some specific substance must be formed in cultures of *B. coli* before reproduction can reach a maximum. It could hardly be maintained that enough of this substance is carried over with a single bacillus inoculated into 10 c.c. of broth to insure rapid growth, when it is remembered that the bacillus is isolated in fresh broth immediately before transferring. If quantities so minute as this are sufficient, no inhibition of growth would be expected when, as in experiments by the older methods, many hundreds of bacteria may be used in the starter.

A possible explanation of the initial slow growth mentioned in some published results is brought out by the following experiment:

Isolations were made of single bacilli taken from a hanging drop in which the bacteria, still actively motile, had assumed the small, short form characteristic of the *B. coli* in older cultures. On the same cover-glass and under the same conditions larger units, taken from young actively dividing cultures, were isolated. Each of these two kind of units, if plated, would give a single colony and be counted as one. At the end of 75 to 80 minutes the hanging-drop cultures formed from the different types of bacilli were stained and counted.

The single bacilli from the older hanging drops had formed, in two cases noted, only one large unit each, and in no drop more than five pieces, while the bacilli from the younger hanging drop had formed from 14 to 20 pieces, each capable of forming a colony in gelatine or agar. So a series in which the beginning and end numbers are determined by plate cultures would show an apparent initial period of slow growth if there were any bacilli of the small type in the starter. Even if the starter were taken from a very young culture there would likely be a proportion of these small forms present which would require, possibly, the time of several generation periods before they grew to a size sufficient for separation in the new medium. Experiments in which the numbers in the starter are estimated by direct observation of stained bacteria instead of by plate cultures would also show the apparent slow initial growth, due to the cause mentioned above. This apparent delay in growth might be greater in these experiments, for, since a considerable number of bacteria must be formed in a culture before they may be estimated by staining, older cultures must be used as starters. The initial delay then, according to my experiments, is due not to a cessation of growth, but to the time required by the shorter elements characteristic of older cultures to assume a size sufficient for division into two new units. I use the term "division" for separation. It is probable that several partition walls may be formed before separation takes place.

I do not say that this explanation alone can account for the initial slow growth described by various authors, for I have not repeated all their experiments with the same organisms they use and under the same conditions. I can only say that in my experiments with *B. coli* there was no evidence whatever of this initial slow growth, when actively dividing bacteria were selected at the start. Some experiments conducted with *B. typhosus* and *B. subtilis* in the warm box (see below) indicates clearly that an initial period of slow growth is lacking in these species also.

Summing up all possible sources of error, it may be considered conservative to estimate the probable error of the determinations approaching the optimum temperature, that is, between 35° and 45°, as little exceeding 5 per cent or one minute in the generation time.

For temperatures much above or below these limits the error is much greater. In this estimate I refer to the most reliable minimum value, the ones marked with the asterisk in Table 1.

The curve plotted below is a recapitulation of the results obtained by all methods. The values are taken largely from the numbers

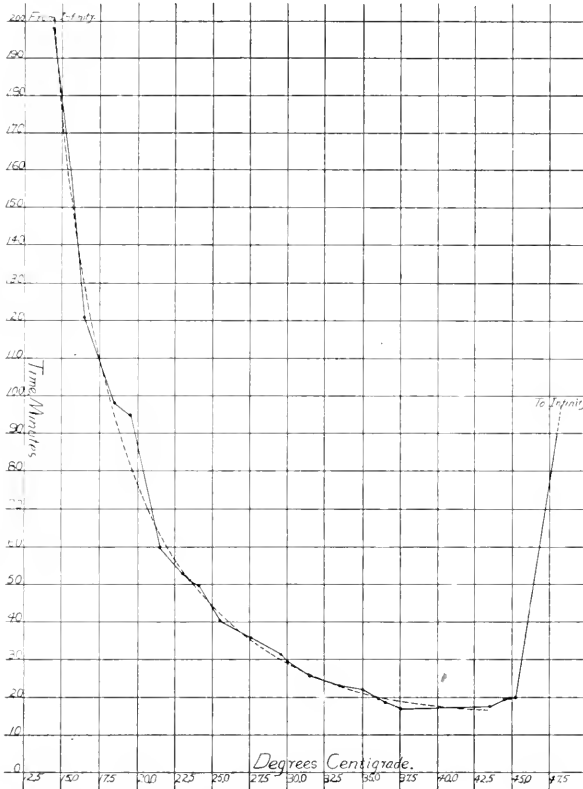


CHART 1.—The growth-rate of *B. coli* at different temperatures. The broken line approximates a curve plotted along the center of gravity of the points determined.

of the first table marked with the asterisk, but some values are taken from other tables also. In the ordinates and abscissae are plotted the time and the temperature respectively. The portion of the curve above 45° and below 12.5 is omitted since the determinations at these temperatures were irregular and uncertain.

The broken line on the chart approximates a curve plotted along

the center of gravity of the points determined. This line does not, apparently, correspond to any simple curve, but the portion extending as far as  $45^{\circ}$  approximates the quarter of an ellipse. The long diameter of this ellipse, owing to difficulties in obtaining exact data at lower temperatures, is almost impossible to determine, so no attempt is made to definitely determine its nature. The graphical presentation in the chart represents the data obtained probably better than an attempted formulation of an equation for the curve or any part of it, and further mathematical considerations will be deferred until further data on this and other species are obtained.

It is observed that the generation time, beginning at infinity, diminishes rapidly at first, then more gradually, and finally reaches a minimum at about  $37^{\circ}$  C. From that point there is but little change until about  $46^{\circ}$  is reached when the rate increases rapidly to reach infinity at  $50^{\circ}$ .

The results shown in this curve tend to confirm the conclusion of Müller<sup>1</sup> that no fever temperature likely to occur in the human body can be high enough to directly inhibit the growth of pathogenic bacteria.

It may be asked how *B. coli* came to be adapted to a relatively rapid growth at temperatures far above body heat and above the temperatures occurring during the warmest summer weather. The explanation may possibly be that this species had adapted itself to growth in the higher temperatures occurring in fermenting masses of organic material. It is certain that in this species there is only a narrow margin between the point at which the maximum rate of reproduction ceases and the thermal death point of a large proportion of the individuals. Even at  $45^{\circ}$  growth was less regular, and there is an increase in the proportion of bacteria which fail to grow. It is possible that bacilli might be isolated which, under the most favorable conditions, would show at temperatures above  $45^{\circ}$  the rapid rate characteristic of temperatures below  $45^{\circ}$  and that the maximum rate of reproduction is closer to the thermal death point than is indicated in my experiments.

These experiments indicate also that in presumptive tests for *B. coli* in drinking water, the incubation temperature should not exceed

<sup>1</sup> *Ztschr. f. Hyg.*, 1895, 20, p. 245.

45° C. The following experiments were carried out to test these temperatures. Water, polluted by sewage, was plated in lactose litmus agar and incubated at 46°3, 47°3, and at 48°3. Controls were incubated at 37°. The controls showed by far the larger numbers of presumptive coli colonies in all three experiments.

From the lowest temperatures tested to about 40° the bacilli were for the most part actively motile. In some hanging drops and in test-tube experiments motility apparently continued actively when the temperature was too low for reproduction. Above 40° the large majority of the bacilli were non-motile, though the rate of reproduction was as rapid as with the motile bacilli at lower temperatures. Occasionally, at temperatures below 40 degrees motility would be absent during the whole time of an experiment, with no apparent diminution of the growth rate. Apparently there is little or no correlation between motility and the rate of reproduction in this species.

The morphology also varied somewhat with the temperature. In freshly growing cultures at temperatures below 40° there was a tendency to break up into relatively long units. In the higher temperatures, especially 45°, there was a tendency to irregularity. Some individuals tended to form long filaments while others, in the same drop, it might be, broke up into very small elements. The short elements tended at these higher temperatures to break up into new elements like themselves, thus resembling the units formed in older cultures at lower temperatures.

The media used in these experiments have been various sorts of beef broth made from meat infusions. The most of the experiments were made with the ordinary beef peptone broth, in some cases plain, and in others modified by the addition of 1 per cent of glucose. It was found that the addition of the sugar made very little difference. The omission of salt made no appreciable diminution in the rate. Infusions of finely chopped lean meat were used in all cases. Broth from meat taken immediately after the slaughter of the animal gave no better results than that made from meat for some time preserved in cold storage. All broths used were acid to phenolphthalein, but various degrees of acidity were tested. The best results were obtained with a broth about 1 per cent acid. Considerable acid, even to 2 per

cent, made but little difference in the rate. Various combinations of beef broth with sterile unheated human serum, with the juices of liver unheated, and extract of muscle unheated, gave no more rapid rate. There was apparently a less rapid rate in fresh human serum in broth than when this serum had stood in the broth for some days. No attempt was made to carefully compare the values of different media further than to ascertain what media gave the optimum conditions. It is quite possible that a medium may be found which would give a still lower generation times than those which I have determined.

All of the experiments described above were made with *B. coli*. It is recognized that another species, or even another race of *B. coli*, might give a very different temperature curve. Little was done with other species of bacteria in my experiments except a few determinations on *B. typhosus* and on *B. subtilis*. The last species was chosen to ascertain if the reproduction rate of a comparatively large species equals that of smaller species. In the warm box and in the hanging drops *B. subtilis* gave a generation time about 20 minutes at nearly 37°. One race of typhoid at 36.6 gave in the warm box 27 generations in 720 minutes, a generation time of 26.7 minutes.

In comparing the results obtained in the above experiments with those of other writers we find that our results, as a rule, show a more rapid rate of reproduction and more uniform values of *G*. Buchner obtained for Asiatic cholera grown at 37.7 results ranging from  $G=19.7$  to  $G=40.0$  min. Rahn obtained for *B. coli* a minimum of  $G=37$  minutes at 27°. For *B. fluorescens liquefaciens* he obtained  $G=60$  minutes at 25° and 30.0 minutes at 28°. Hehewerth obtained for *B. coli* at 37° an average value of 23 minutes 24 seconds with a minimum two minutes or so lower. At 22° he obtained for *B. coli* 77 minutes 54 seconds. For *B. typhosus* at 37° he obtained an average value of 33 minutes 24 seconds. Müller obtained for *B. typhosus* at the temperature 37.5–38.1 an average of 32.02 minutes and a minimum of 28.65 minutes. For his bacillus A he obtained the following series:

30 degrees	46 min.	38 sec.
25 degrees	51 min.	24 sec.
6 degrees	7 hrs.	36 min.
0 degrees	19 hrs.	57 min.

For his bacilli B, C, and D, and *B. fluorescens liquefaciens* he obtained somewhat similar ratios.

#### SUMMARY.

The reproduction of *Bacillus coli*, beginning at about 10°, increases rapidly to about 37° C. where it reaches its maximum rate with a generation time of nearly 17 minutes. This rate is nearly constant until the temperature of 45° when it falls rapidly and reproduction practically ceases at 49°.

The initial slow growth described by various authors as occurring when small numbers are transferred to fresh sterile medium does not occur in this species, if an actively dividing bacillus accustomed to the medium is used as a starter.

Under constant conditions the rate of growth remains constant at least as far as the thirty-eighth generation, and all offspring continue to divide at the same geometrical ratio as the parent bacilli.

It is not probable that any body temperature during fever can be high enough of itself to materially alter the rate of reproduction of pathogenic bacteria.

There is with *B. coli* little or no correlation between motility and rapidity of division.



## THE VALUE OF THE AGGLUTINATION REACTION AND OF BACTERIOLOGICAL METHODS IN THE DIAGNOSIS OF GLANDERS.\*

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THE readiness with which the agglutination test can be carried out when compared with the mallein test for glanders has made it the subject of much investigation by those interested in comparative medicine. In attempting to establish the agglutination test as a routine method of diagnosis we were confronted by many questionable cases that proved puzzling to the diagnostician, and although the delicacy and specificity of the reaction seemed to be very well established by many observers it was deemed advisable to undertake a thorough study of the method with the hope of coming to some practical conclusion as to the disposition of these anomolous cases. Therefore while very little that is new is here presented our experience may prove of some interest and value in the practical application of the test. In confirming the results of the test by bacteriological findings some new facts were developed that have not heretofore been mentioned by any of the observers along these lines.

During the course of this work all clinical observations and autopsies have been made and blood specimens furnished by Dr. H. D. Gill, veterinarian for the State Department of Health, New York, Dr. K. S. Silkman, veterinarian for the Department of Health, New York City, and Dr. R. H. Kingston, New York City. Dr. E. P. Miller and Dr. R. E. Pick have also rendered valuable assistance in the routine testing.

After following the application of this reaction for over two years, during which time about 2,500 horses of all classes have been tested, the results, in our opinion, are not so conclusive from a practical standpoint as to make it a means of absolute diagnosis.

This difference of opinion is not wholly due to the use of different methods, as at first might be assumed, for while the microscopical

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method has been relied upon for the routine work, the macroscopical method of Schütz and Meissner<sup>1</sup> has been systematically employed as control in a large number of cases, and we have found the same exceptions occurring with both methods. Some of the points of difference between the two methods and our reason for considering that the microscopical method gives the best results will be dealt with later.

Pokchichevski<sup>2</sup> in 1902 found that normal horse blood would agglutinate the *B. mallei* in dilutions of 1:300, while the blood of horses infected with glanders would agglutinate it in dilutions of 1:500 and above.

Afanassjeff,<sup>3</sup> Schnürer,<sup>4</sup> and Bonomé<sup>5</sup> confirmed these results, while Hutyra,<sup>6</sup> inducing experimental glanders in horses, found that the agglutination index ranging from 1:100 and 1:300 before injection of *B. mallei* was raised to 1:1,000 and 1:2,000 several days after inoculation.

Schütz and Meissner of the Pathological Institution, Berlin, in 1905 undertook an extensive series of observations upon the subject. A number of horses were given experimental glanders and in from 6 to 12 days after infection the agglutinating index of the blood serum of these horses had increased from 1:300 to 1:2,000 and 8,000. Subsequent autopsy of these showed the typical lesions of glanders in the lungs and other organs. In the course of their work 2,209 horses were tested. This number included normal horses, horses having glanders, and those ill of other diseases than glanders.

The following table gives a very good average of the results they obtained, and from these tables it will be seen that 48 horses out of 238 which were glanders-free reacted at a point that is considered by them to be diagnostic of glanders. Out of 37 horses having various diseases other than glanders they give 10 as reacting between 1:500 and 1:1,000. These tables are quoted in order to show that although Schütz and Meissner used the macroscopical method

<sup>1</sup> *Wissenschaftliche u. Prakt. Tierh.*, 1905, 31, p. 353.

<sup>2</sup> *Centralbl. f. Bakt.*, 1902, Ref. 31, p. 507.

<sup>3</sup> *Ibid.*, 1901, Ref. 29, p. 41.

<sup>4</sup> *Ibid.*, 1905, 30, p. 180.

<sup>5</sup> *Ibid.*, 1905, 38, pp. 601-732.

<sup>6</sup> *Ztschr. f. Tiermedizin.* 1902, 2, p. 8.

throughout their work, while we used the microscopical method, yet they find that the normal index may sometimes be as high as 1,000, while on the other hand a certain percentage of horses having glanders will react as low as 500.

TABLE 1.

AGGLUTINATION OF *B. mallei* BY SERUM OF NORMAL AND DISEASED HORSES. (SCHÜTZ AND MEISSNER.)

Glanders-Free						Glanders					
1:300	400	500	600	800	1,000	500	600	800	1,000	1,500	2,000
145	36	17	15	6	2	2	4	6	11	6	6
221 (25 dead)						34					
(255 horses.)											

TABLE 2.

AGGLUTINATION OF *B. mallei* BY SERUM OF NORMAL AND GLANDERED HORSES.  
(SCHÜTZ AND MEISSNER.)

Glanders-Free						Glanders					
1:300	400	500	600	800	1,000	500	600	800	1,000	1,500	2,000
5	4	2	1	4	1	..	2	2	4	9	2
17						19					
(36 horses.)											

We have also found that it is not possible to determine an absolute boundary between the normal and the infected horse that will not at times prove unsatisfactory; our experience has led us, however, to adopt the usual index of 500 as the lowest to indicate infection. The exceptions to this will be given later.

In considering the method to be used in making these tests we found the microscopical method to be more satisfactory than the method of Schütz and Meissner for the following reasons:

First, Schütz and Meissner submit the emulsion of the bacillus to 60° of heat for two hours; this tends to break up the bodies of the bacilli and to release the precipitable substances, so that the phenomenon would partake more of the nature of a precipitin rather than of an agglutinin reaction. As is well known, the precipitins are not formed in the blood to the same extent as the agglutinins, so a different range of index would be required for the two reactions; the index limit for precipitins being considerably lower than for

agglutinins in the same serum. The confusion of these two reactions does not seem to us to add to the uniformity of the test, as the relation between precipitin and agglutinin is not constant but varies in different immune sera.

Secondly, while the readings of the microscopical method average higher than the macroscopical method, yet by the former method we find fewer horses exhibiting clinical and other symptoms of glanders falling below 1:500 in their agglutinating index, thus rendering the latter method less reliable where more definite indications are absent. The microscopical will therefore bring under suspicion a greater number of perhaps questionable horses to be tested with mallein and thus fewer actual cases will be overlooked.

The following table presents a fair average of the difference observed in all cases tested by the two methods. As we rely upon the agglutination test chiefly as an indication for the use of mallein it is quite obvious that in such instances the advantage lies with the microscopical test.

TABLE 3.  
THE SERA OF FIVE HORSES TESTED BOTH BY THE MICROSCOPICAL AND MACROSCOPICAL METHODS.

Number	100	200	500	1,000	2,000	5,000	10,000	Mallein Test	Final Disposition
9 } Mic.....	+	+	+	+	+	+		Negative	Working
/ Mac.....	+	+	-	+	+	+		"	"
10 } Mic.....	+	+	+	+	+	+	+	Positive	Destroyed
/ Mac.....	+	+	+	+	-	-	-	"	"
A } Mic.....	+	+	+	+	+	+	+	"	"
/ Mac.....	+	+	+	+	-	-	-	"	"
B } Mic.....	+	+	+	+	+	+	+	"	"
/ Mac.....	+	+	+	+	+	-	+	"	"
C } Mic.....	+	+	+	+	+	+	-	"	"
/ Mac.....	+	+	-	-	-	-	-	"	"

It is claimed by Bonomé that mallein increases the index. This change we found not to be constant, but on the other hand to vary. Horses tested the first 24 hours after the injection of mallein gave an agglutinating index that was slightly lowered, but tests made subsequent to this were so irregular that we were unable to convince ourselves that the changes were due to the effect of mallein instead of the progress of the disease.

A series of tests made upon four horses show the variation in the

agglutination reaction that may sometimes occur. In the table below a regular increase is observed in three, while the third shows an irregular rise and fall. This condition occurs constantly in experimental animals immunized with various organisms.

TABLE 4.

FIRST TEST			SECOND TEST		THIRD TEST	
Horse	Agglutination	Mallein	Agglutination	Mallein	Agglutination	Mallein
1.....	500	Positive	2 weeks later, 2,000	Positive	2 weeks later, 10,000	Positive
2.....	5,000	Positive	2 weeks later, 10,000	Positive	.....	.....
3.....	2,000	Good	5 weeks later, 5,000	Good	10 days later, 2,000	.....
4.....	2,000	Good	5 weeks later, 5,000	.....	5,000	Good

Meissner and Schütz found that the blood of one horse with lymphangitis, out of four tested, agglutinated at 1:1,000 and two at 1:800. They state that this is the only condition, not glanders, that will give such a high reaction.

Nine horses with lymphangitis were tested by us, and mallein was given to four.

2 agglutinated at 1:2,000, mallein not given

1	"	"	1:1,000	"	"	"
1	"	"	1:5,00	"	"	"
1	"	"	1:100	"	"	"
1	"	"	1:5,000	"	"	"
1	"	"	1:5,000	"	negative	
1	"	"	1:1,000	"	"	
2	"	"	1:2,000	"	positive.	Destroyed for glanders.

The question that might arise here is whether or not there was a latent glanders in those cases where the reaction is high. More extensive research is necessary before any conclusion can be reached, but the fact that two horses apparently free from glanders reacted to mallein is suggestive. The mallein was not given until the horses had fully recovered from the attack of lymphangitis.

The practice has been followed of taking a stable where the horses are permanent and testing the blood of all horses, then giving mallein to as many as possible and keeping the stable under observation for some time.

In one stable consisting of 32 horses where glanders had occurred the reactions were as follows:

Number of Horses	Agglutinating Index	Number Reacted to Mallein
4	500	2
12	Between 1,000-2,000	6
7	" 5,000-10,000	3
9	Below 500	None

In another stable containing sixty horses the results were as follows:

Number of Horses	Agglutination Reaction	Number of Horses Tested with Mallein	Number Reacted to Mallein
9	500	3	2
9	1,000	6	3
7	2,000	4	4
35	Below 500	2	2 negative

In this case 9 out of 13 horses agglutinating above 500 reacted to mallein. We were not able to follow this stable.

The table below gives the results of the agglutination test of 49 horses. All the horses with the exception of 16 which showed definite characteristic symptoms of glanders were tested with mallein.

TABLE 5.  
AGGLUTINATION REACTION OF HORSES TO *B. mallei*.

	Below 500	500	1,000	2,000	5,000	10,000	20,000	Total	Note
No. of horses		3	2	10	7	3	1	26	Reacted to mallein test; clinical symptoms suspicious
" " "		2	5	4	3	..	2	16	Not tested with mallein, clinical symptoms characteristic
" " "		..	..	..	1	..	..	1	Negative to mallein; clinical symptoms characteristic
" " "		2	..	3	1	..	..	6	Negative to mallein; clinical symptoms not characteristic

In a stable of 16 horses three of which had died of glanders we were able to follow up the agglutination and mallein reactions by autopsy. Our observations extended over a period of four months. Table 6 shows the relation of the agglutination test and the mallein reaction and autopsy findings.

In case No. 1, although all definite symptoms are absent, the very marked and sudden rise of agglutinins would indicate very strongly a glanders infection. Pus from an external ulcer did not develop the Straus reaction in guinea-pigs.

No. 6 probably comes under the exceptional cases that show a lowered index in the last stages of the disease.

We have found about three cases per 1,000 falling below 500 that have shown definite symptoms of glanders and are in the last stages of the disease.

TABLE 6.  
RELATION OF AGGLUTINATION AND MALLEIN TESTS TO AUTOPSY FINDINGS.

HORSES	OCTOBER		NOVEMBER		DECEMBER		JANUARY		RESULTS OF AUTOPSY
	Agglutination	Mallein	Agglutination	Mallein	Agglutination	Mallein	Agglutination	Mallein	
1.....	.....	.....	200	.....	200	Neg.	5,000*	Neg.	No lesions on autopsy
2.....	5,000	Neg.	.....	.....	5,000	.....	10,000	Pos.	Died of glanders
3.....	5,000	.....	5,000	.....	10,000	Pos.	.....	.....	Killed; lesions characteristic of glanders
4.....	5,000	.....	2,000	.....	10,000	Pos.	.....	.....	Killed; lesions characteristic of glanders
5†.....	.....	.....	.....	.....	500	Pos.	.....	.....	Killed; lesions characteristic of glanders
6‡.....	.....	.....	.....	.....	†	Pos.	.....	.....	Killed; lesions characteristic of glanders
7.....	.....	.....	500	.....	500	Pos.	.....	.....	Killed; lesions characteristic of glanders
8.....	.....	.....	.....	.....	10,000	Pos.	.....	.....	Killed; lesions characteristic of glanders
9.....	.....	.....	.....	.....	Neg.	Neg.	.....	.....	Killed; no lesions
10.....	.....	.....	.....	.....	10,000	Pos.	.....	.....	Killed; lesions characteristic of glanders
11.....	.....	.....	2,000	.....	1,000	Pos. on 2d test	.....	.....	Killed; lesions characteristic but not marked
12.....	.....	.....	5,000	.....	5,000	Pos.	.....	.....	Killed; lesions characteristic of glanders
13.....	.....	.....	.....	.....	1,000	Pos.	.....	.....	Died of glanders

\* This horse developed an ulcer on the leg and the second day the blood was tested with the results given above.

† Skin lesions appeared on the lower maxillary a few hours before killed.

‡ This horse had been immunized for a period of two years with typhoid, dysentery, pneumococcus cholera, and tetanus respectively.

One of the possible causes of a lowered index in the final stages is the invasion of other organisms which, supplanting the infecting organism, form agglutinins or other anti-bodies which affect the agglutinins, as was shown by Klein who caused the agglutinins in rabbits immunized to *B. typhi* to disappear by discontinuing the inoculation of the typhoid bacillus and substituting that of cholera.

In estimating the reliability of the agglutination reaction as an independent means of diagnosis several factors must be taken into consideration. In reviewing the results as given in the above tables the weight of evidence certainly seems to indicate that a reaction above 500 indicates a glanders infection, either latent or active according to the other symptoms present.

This conclusion as to the specificity of the reaction is further

borne out by the absorption test and animal inoculations. A number of organisms found in the superficial ulcers, in material from the lungs, glands, and nasal secretions associated in many cases with *B. mallei*, were isolated. These failed to absorb out the agglutinins; they also failed to raise agglutinins for the *B. mallei* when inoculated into rabbits and only one organism produced agglutinins for itself. Therefore such organisms, as far as tested, could not give rise to the agglutinins for *B. mallei*.

Hutyra found in experimental glanders that the agglutination reaction appeared before the mallein reaction, thus making a more delicate test. So when the reaction is 500 and above, becoming more certain as it increases, it seems justifiable to assume that even in the absence of other symptoms a slight infection exists which may remain latent for some time and later recover or go on to a full development of the disease. The similarity of this disease, in many of its phases, to tuberculosis is marked and there is no reason why we should not assume that recovery may take place.

It is well known that even after reacting to mallein a horse may remain in good condition for some years. One horse whose history we were fortunate enough to obtain had two years previously reacted to mallein, but as he was in good condition he was not destroyed. When brought to our notice he gave a mallein reaction and a blood reaction of 2,000, and on autopsy old lesions were found as well as fresh ones. In spite of these very suggestive results we find a number of horses in apparently good health giving a high agglutination reaction to *B. mallei* and giving no other evidence of infection. The destruction of such horses, considering the number of them, does not seem to be justifiable at present. We have had some of these high reactors under observation for the past 18 months. They have continued working and are apparently normal and glanders has not occurred during this time among the other horses in the same stable. It does not seem practical to destroy such horses unless some compensation can be offered their owners, as it would sometimes mean a loss of 25 to 40 per cent. But it is possible often to watch these cases and have them tested from time to time.

The serum in human cases of glanders as far as tested generally reacts in dilutions of 1:1,000 and 1:2,000.



McFadyycan<sup>1</sup> found that blood of patients having diphtheria and scarlet fever would agglutinate the glanders bacillus in dilutions of 1:50 and 1:100. We found that normal human serum did the same, as did also serum from patients having typhoid fever and tuberculosis, while the serum of cases of glanders infection agglutinated as high as 1:1,000 and 1:2,000, unless the course of the disease was very rapid, extending over a period of three to five days. In such cases sufficient length of time has not elapsed for the agglutinins to be formed and the severe intoxication would prevent the cells from reacting.

Our failure always to recover the organism from material obtained from doubtful cases of glanders led us to make the following observations concerning the readiness with which the *B. mallei* is supplanted by the various organisms found in the glandered tissue. This was tested by growing the *B. mallei* in combination with several of these organisms; also growing each organism separately and combining just before inoculation into pigs, and plating; and growing them together in a mixture. The pigs inoculated with the 24 hours' mixed growth gave a negative Straus reaction, and the *B. mallei* was not recovered from the plates made from this mixture, while the separate cultures combined at time of inoculation produced an early Straus reaction and the *B. mallei* was readily isolated from the plates. This would explain the failures, that sometimes occur, to obtain bacteriological evidence of the presence of the glanders bacillus in the suppurative lesions of a horse that has glanders.

In the inoculation of suspected glandered material into guinea-pigs an anomalous Straus reaction was constantly encountered which was quite misleading. After 48 to 72 hours, following intraperitoneal injection, there would develop an apparent beginning Straus reaction, which would remain stationary or retrogress and finally disappear entirely. There would be sometimes a bilateral and sometimes a unilateral descent of the testicle with marked enlargement; generally the organ was reducible under pressure but slipped back as soon as the pressure was removed. This was more noticeable in the bilateral cases. Autopsy revealed an enlarged testicle hyperemic with more or less sclerosis and marked injection of the scrotal tissue

<sup>1</sup> *Jour. Comp. Path. and Therap.*, 1896, 9, p. 322.

and sometimes adhesions, but no abscess formation or caseation. Nicolle<sup>1</sup> found that heated cultures of *B. mallei* produced anomalous reactions and from his experiments was led to describe the following successive symptoms: first, paralysis of the testicle which ceases to return spontaneously into the abdomen, edema of the scrotal tissues, definite fixation and abscess formation, followed by ulceration. With a non-fatal dose of the killed culture he was able to bring about the first stage of the reaction. We were able also to bring about this reaction not only with killed cultures but with suspected material and by the intraperitoneal injection of the filtered products of *B. mallei*, unheated, and of three days' growth; also with mallein; and in three instances with the serum of animals having undoubted glanders.

This last phenomenon is, according to our experience, rare, and would seem to depend upon the large amounts of endotoxins in the blood of the animal. The organism was not found in the blood in these cases. The animals tested were all severe cases of glanders, as proven by the mallein test and autopsy. Serum from normal horses failed to give this reaction. The filtered products of pyocyaneus and other organisms frequently associated with the glanders bacillus also fail to react. This suggests the possibility of the action being due to a toxin or endotoxin which is selective in its nature, acting upon this peculiar nerve structure, inducing the paralytic stage, and thus rendering the organ sensitive to the subsequent action of the bacillus itself in producing abscess.

This dual action seems to be emphasized by the fact that at times the infection may be more general or localized in other tissues, suggesting the possibility of a variation in the amount of toxin introduced at the time of inoculation. In the female, where this same peculiar nerve structure does not exist, the infection is general or localized in the glandular or peritoneal tissue.

The examination of the nasal secretion may sometimes furnish evidence of the existing disease but the presence of other organisms overgrowing *B. mallei* makes the test reliable only when positive. It should be resorted to, however, in all doubtful cases as it offers one more means of diagnosis. In obtaining material from the mucous membrane of the nasal cavity we use a long sterile swab made of

<sup>1</sup> *Brit. Med. Jour.*, 1906, 2, p. 1569.

heavy iron wire and wrapped with cotton which enables us to obtain material rather high up in the nostril.

#### SUMMARY.

1. The serum of horses reacting with *B. mallei* in dilutions of 1:1,000 and above indicates a glanders infection which may be latent or active according to the accompanying symptoms and reaction to mallein.

2. Horses giving a reaction of 1:500 to 1:1,000 are to be suspected and should be tested with mallein; if no reaction occurs and the horse is apparently in healthy condition, it may be considered free from glanders.

3. A few cases, about 3 in 1,000, will give a reaction below 500 and yet respond to the mallein test. Such horses generally give clinical evidences of glanders.

4. Horses giving a positive reaction, that are apparently in good health and fail to react to mallein, do not seem a menace to other horses about them, but should be kept under observation and tested from time to time both for the agglutination and mallein reaction. The secretion of the throat and nose of such horses should be examined for the presence of the glanders bacillus.

5. The presence of other bacilli in the tissues of a horse dying with glanders may interfere with the appearance of a Straus reaction in a guinea-pig inoculated with such post-mortem material.

6. When a guinea-pig is inoculated with post-mortem material which has become saturated with toxins and contains only a few living glanders bacilli, due to the presence of other invading organisms of a pyogenic nature, the paralytic stage of the Straus reaction only results.

7. In a few instances this paralytic stage may follow the inoculations of serum from horses having well-developed glanders.

8. This paralytic stage is brought about by the inoculation of mallein, filtrate of *B. mallei* broth culture, but not by filtrate from pyocyanus broth cultures.

9. In human cases a reaction above 100 would undoubtedly mean glanders.

## A COMPARISON OF PRACTICAL METHODS FOR DETERMINING THE BACTERIAL CONTENT OF MILK.\*

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THE American Public Health Association appointed a committee in 1905 to formulate standard laboratory methods for the bacterial examination of milk, following the example set by a similar committee on water analysis. The methods of milk examination in vogue at present are so widely different in the different laboratories that results cannot be compared. These different results are due to at least four factors, viz., the medium employed, the temperature of incubation, the period of incubation, and the degree of shaking before plating.

The media usually employed are agar or gelatin. There are undoubtedly some arguments in favor of the use of gelatin, but the difficulty in handling gelatin plates, especially in warm weather, and the great loss occasioned by the presence of liquefying colonies probably more than counterbalance the advantages. The addition of litmus and dextrose or lactose is a common practice.

The temperature of incubation has been the subject of considerable discussion, inasmuch as some workers favor incubation at 37° C., others at 20°. Since certain bacteria multiply more rapidly at 37° than at 20°, the former temperature has been often employed, so that in many laboratories counts are made after 24 hours' incubation at 37°. It is true, however, that at 20° a higher count can be obtained than at 37°, and this fact seems to be of sufficient importance to advocate incubation at the lower temperature. It is generally conceded that at 37° multiplication is more rapid than at 20°, so that plates can be counted at an earlier date.

Finally the numbers may vary with the degree of violence used in shaking milk dilutions preparatory to plating, as has been shown recently by Rosenau and McCoy.<sup>1</sup>

The points just mentioned explain to some extent the varying

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<sup>1</sup> *Jour. Med. Res.*, 1908, 18, p. 165.

results reported from different laboratories on the same milk. Within our own experience these results have varied enormously. The desirability of uniform methods is therefore emphasized, not only from a scientific point of view, but uniformity of methods should also be made a basis in the framing of ordinances for the regulation of milk supplies.

The ordinary bacteriological examination of milk consists chiefly of the colony count. Some significance has been attached to a study of bacterial species, a procedure which eventually may be of great value. The length of time required for a study of this nature and the uncertainty in interpreting results owing to our imperfect knowledge of microorganisms are the most serious objections to an extended study of species in milk. The total colony count therefore gives at present the most valuable control of milk and the efficiency of this method may be enhanced by establishing uniform methods. In water examinations we have different conditions from those obtaining in milk. The significance of fecal bacteria in water is well understood, total numbers being of secondary importance. Fecal bacteria in milk do not as a rule originate from human beings and therefore the significance of their presence is not the same as in water. The presence of fecal bacteria in milk signifies chiefly contamination with cow feces, in other words filthy methods in milking.

Bacteria as a rule are capable of enormous multiplication in milk. In water on the contrary they multiply but little. A high bacterial count in milk then has some decided significance. From this point of view it appears that numbers in milk are of more value than the differentiation of particular species.

To judge from the origin of bacterial contamination in milk we may distinguish several more or less distinct groups of bacteria, viz.:

1. Those gaining access to milk from the air.
2. Fecal bacteria.
3. Bacteria from contact with the hands and clothes of milkers.
4. Bacteria dropping into the milk from the cow during milking.
5. Bacteria in the utensils.
6. Pathogenic bacteria.

Fecal bacteria are chiefly acid formers and probably most bacteria originating from the cow and utensils are of this nature.

Many air bacteria do not form acid and we can thus by making a differential count of acid and non-acid forming colonies gain some insight into the conditions which have governed the production and handling of the milk. Pathogenic bacteria, if present, are usually in relatively small numbers, so that they can be detected with great difficulty.

The experiments reported in this paper were undertaken in order to put the considerations outlined above on a definite basis. The medium used was sugar-free<sup>1</sup> meat extract agar<sup>2</sup> with addition of 1 per cent dextrose in one set of plates and 1 per cent lactose in another set. A litmus solution prepared from Merck's pure extract of litmus was poured on a Petri dish before the milk dilution and the liquefied agar were added. Care was taken not to allow the milk dilution and the litmus to mix before the liquefied agar was added, on account of the alleged germicidal action of litmus. The samples were purchased from various dealers and dilutions of 1:1,000, 1:10,000, and 1:100,000 prepared. The dilution flasks used for this purpose were filled with 100 c.c. of distilled water and it was assumed as a result of frequent tests that they contained 99 c.c. after having been sterilized in the autoclave. Particular attention was paid to shaking all mixtures of milk and water in as uniform a manner as possible so as to break up the clumps of bacteria in the milk equally in all samples. Two sets of plates were prepared from each sample, two with lactose and two with dextrose. Then one from each set was incubated at 37° and one of each at 20°. The incubator for the low temperature, in which these plates were kept, is of very simple construction. An ordinary ice chest is kept cool by water from Lake Michigan circulating through a series of pipes and an incandescent electric light furnishes the heat. By a simple device the electric current is disconnected automatically as soon as 20° is reached and if the temperature falls below this the connection is re-established. The temperature was noted twice daily and the variations were less than one degree each way.

<sup>1</sup> Sugar-free meat extract agar was used in order to eliminate the error caused by the presence of muscle sugar in comparing dextrose and lactose agar. For routine work the small amount of muscle sugar in meat extract is of no consequence.

<sup>2</sup> Composition: 3 grams Liebig's extract of meat, 10 grams Witte's peptone, 15 grams agar in 1 liter of water. 8 c.c. of this medium were filled into each tube, so that after adding 1 c.c. milk dilution and 1 c.c. litmus solution the amount of agar was 1½%. Reaction 1 per cent acid.

TABLE I.  
SUMMARY OF RESULTS OBTAINED FROM EXAMINATION OF 46 SAMPLES OF MILK.

DAYS	ACID COLONIES				NON-ACID COLONIES				TOTAL COUNTS			
	Dextrose Agar		Lactose Agar		Dextrose Agar		Lactose Agar		Dextrose Agar		Lactose Agar	
	20°	37°	20°	37°	20°	37°	20°	37°	20°	37°	20°	37°
1 .....	0	110,675	100	132,075	902,725	1,653,050	718,100	1,200,850	902,725	1,764,325	718,200	1,422,025
2 .....	687,400	1,605,775	454,100	1,114,725	4,677,600	2,115,050	4,841,400	2,586,525	5,305,000	3,721,725	5,208,500	3,701,250
3 .....	2,797,975	2,022,175	1,726,775	1,203,575	6,382,150	2,217,475	7,424,150	2,853,425	9,180,125	4,239,650	9,150,925	4,117,000
1 .....	0%	6%	0%	0%	100%	94%	100%	91%	.....	+861,600	.....	+794,725
2 .....	15%	43%	8.5%	30%	87%	57%	91.5%	70%	+1,643,275	.....	+1,597,250	.....
3 .....	30%	48%	19%	31%	76%	52%	81%	69%	+4,945,475	.....	+5,033,925	.....

Spreaders: At 20° = 5%; at 37° = 15%.

In counting the colonies those plates were selected which showed the presence of 200 to 500 colonies; the acid forming colonies were counted separately. The total count was also recorded so that a definite ratio between acid and non-acid forming colonies could be established. The counts were repeated on each of three successive days. After this period the number of colonies still increases, but the increase is slow and the total is not increased very much although more pronounced at 20° than at 37°. In the Thirteenth Annual Report of the Board of Health of the Town of Montclair, N. J., it is stated that milk plates were incubated for seven days at room temperature, and in a private communication Dr. Baldwin stated that the count after three days at room temperature gave perfectly satisfactory results.

Table 1 here given is a summary of the results obtained from the examination of 40 samples of milk in the above-described manner. If we compare the total counts with reference to the temperature of incubation we find that in dextrose agar the number after one day is larger at 37°, after two days the number is higher at 20°, and after three days the number of colonies at 20° is about double that at 37°. In lactose agar conditions are practically the same.

As to the number of acid colonies, at 20° there are no acid colonies in either dextrose or lactose agar after 24 hours. After two days the number of acid colonies in both dextrose and lactose agar is considerably larger at 37° than at 20°, but this proportion is reversed after three days, the acid colonies developing at a more rapid rate after two days at 20° than at 37°. The proportionate number of acid colonies, as compared with the total number of colonies developed, is smaller at 20°, however, than at 37°, which would seem to indicate that acid formers which are chiefly of fecal origin are favored at 37° at the expense of non-acid formers. It is obvious then that at 20° we obtain a more complete representation of the groups of bacteria gaining access to milk than at 37°. And since the actual number of acid formers is really higher at 20° than at 37° after three days, it seems reasonable to assume that they are represented in fair proportion.

The relation of acid forming colonies to non-acid formers in



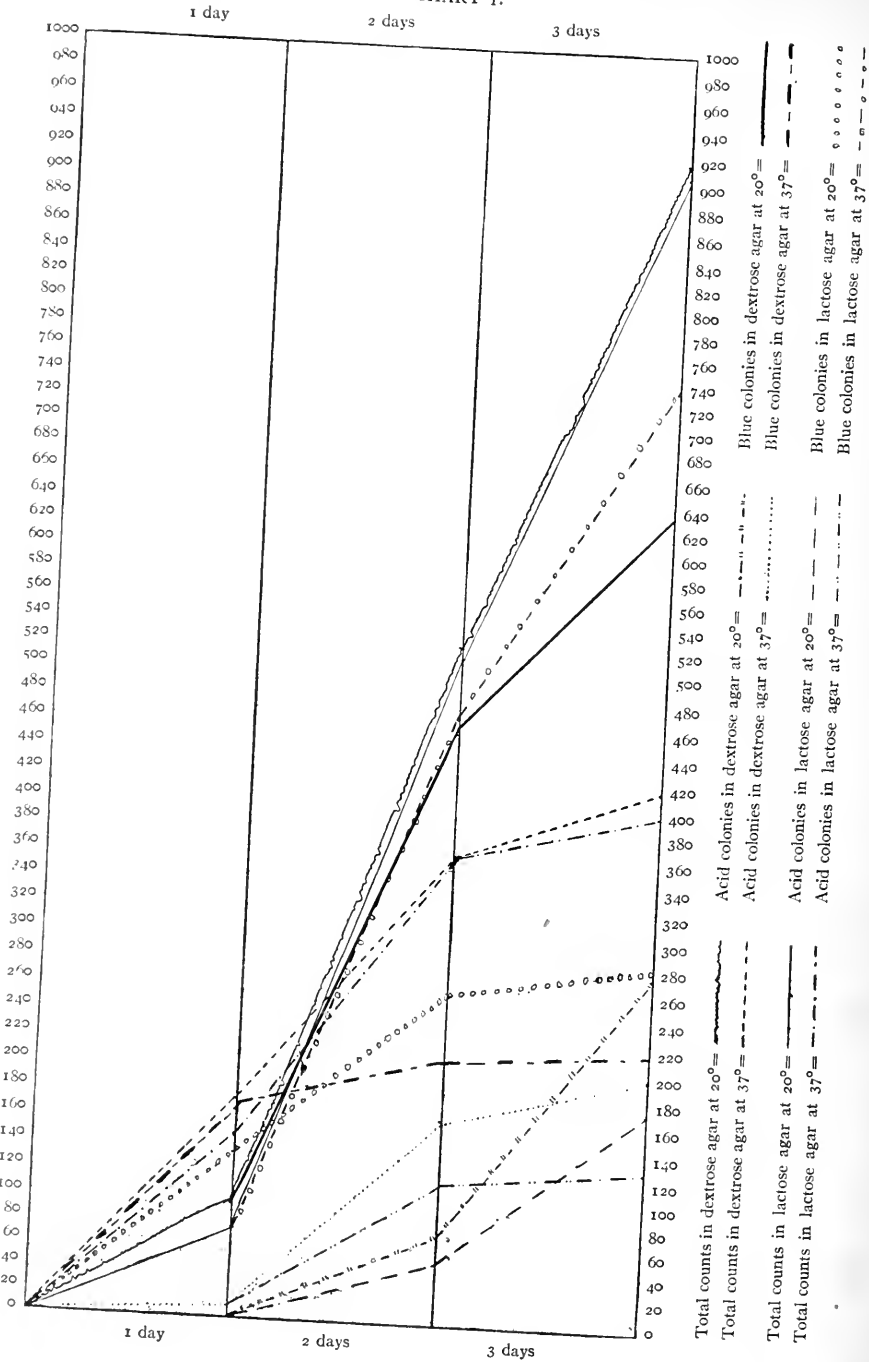
dextrose and lactose deserves special notice. There is in some samples a decided relative decrease of non-acid colonies after two and three days at both temperatures in lactose agar. This phenomenon is undoubtedly due to a fact which has been noted repeatedly, namely, that some milk bacteria of the *B. aërogenes* type form red colonies at first and then later these colonies assume the blue color again. This phenomenon was not observed in dextrose agar. We conclude from this observation that dextrose is to be preferred as an addition to the medium to lactose.

The number of plates lost by the overgrowth of spreading colonies is three times larger at 37° than at 20°. The loss might be prevented partially by using porous-cover petri dishes, but these covers render counting more difficult and inaccurate and the jelly is more liable to become dry. The insertion of a piece of filter paper under the cover of a petri dish before sterilization is perhaps a better method for the absorption of condensation water.

One of the advantages then of incubation at 20° over 37° is the larger count obtained. This may not be of prime importance since we cannot hope to demonstrate the presence of nearly all bacteria in milk by ordinary methods. The count after two days at 37° is, however, so much larger than after one day that, if 37° is to be the incubation temperature, the period of incubation should be at least two days. The incubation at 20° after two days gives a higher count than at 37° and a still higher one after three days. Thus the time gained is but one day by using the higher temperature and if the better differential count at 20° is taken into consideration this temperature is surely preferable. We favor the incubation at 20° for three days, therefore, chiefly because more groups of bacteria have a chance to develop than at 37° and this gives us a better insight into the production, handling, etc., of milk.

The results of our experiments are graphically illustrated in the following chart which is self-explanatory. It is shown here very clearly that the numbers of bacteria steadily increase at 37° until a high point is reached after two days, while at 20° the increase is slow for 24 hours and then the high point is reached after three days, although even after two days the numbers at 20° are higher than at 37°. It is also shown that the acid colonies develop more rapidly

CHART 1.



at 37° for two days after which time a higher number is reached at 20°. Finally we note that more acid colonies develop in dextrose agar than in lactose agar.

Several objections to the use of 20° as incubation temperature have been advanced, some of which will be briefly discussed.

1. Pathogenic bacteria do not grow so well at the lower temperature. This is undoubtedly true, but the finding of pathogenic bacteria in milk, as in water, is very difficult and in the majority of cases search for them is unsuccessful, and when successful is the result of long and laborious work. It may safely be stated that under present conditions the probability of finding pathogenic bacteria in milk is so remote that any attempt to find them is entirely out of the question in routine examinations of milk. Incubation at 37° therefore affords no benefit in this respect.

2. Incubation at 20° requires more time than at 37°. It has been shown in this paper that the difference is but one day and this is surely too short a period for which to sacrifice accuracy. A longer period of incubation also necessitates the use of more material and glassware, which, however, does not seem a sufficiently weighty objection to the use of 20° for three days if better results can be obtained.

3. Great stress is laid by some workers on the claim that it is difficult to procure incubators which can be kept uniformly at 20°, especially in summer weather. There is a description of a practical low-temperature incubator in the new edition of William's *Manual of Bacteriology*, which also refers to Rogers' paper describing an electrically controlled low-temperature incubator.<sup>1</sup> This type of low-temperature incubators has been used in several laboratories with entire satisfaction. It is possible to keep an incubator of this nature in any locality where electricity and ice are available.

The following conclusions seem warranted from the results of the experiments reported:

1. Official bacterial standards for milk should include a statement of the methods by which the bacteriological control is to be obtained.

2. For practical purposes incubation at 20° is superior to incuba-

<sup>1</sup> *Centralbl. f. Bakt.*, 1905, Abt. 2, 15, p. 236.

tion at  $37^{\circ}$ , because: (a) a higher count is obtained; (b) a better differential count is obtained.

3. Dextrose is to be preferred to lactose as an addition to the medium.

4. Since milk is usually consumed before the results of a bacteriological examination can possibly be reported, a gain of one or even two days is immaterial. The bacteriological and chemical examination of milk ought to be carried on chiefly with the object of improving the whole milk supply of a commonwealth rather than of punishing individual offenders. To this end the most accurate and scientific method of examination is the preferable one.

## VARIABILITY IN THE DIPHTHERIA GROUP OF BACILLI.\*

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ONE of the most interesting questions in bacteriology at present is that of the mutual relationship borne by the organisms in the so-called "groups." In the case of the diphtheria group the problem is of particular importance because of its bearing on public health service methods, as these naturally must differ in many respects if the group consists of two or more distinct species instead of but one comprised of several types.

The overwhelming majority of bacteriologists at present look upon the group as consisting of at least three definite species, *B. diphtheriae* (Klebs-Löffler<sup>1</sup>), *B. pseudodiphthericus* (Hofmann-Wellenhof<sup>2</sup>), and *B. xerosis* (Kuschbert and Neisser<sup>3</sup>), while opinion is divided in regard to the numerous other types, collectively known as diphtheroids, of which the most important are *B. coryzae segmentosus* (Cautley<sup>4</sup>), *B. diphtheroides* (Klein<sup>5</sup>), *B. clavatus* (Kruse and Pasquale<sup>6</sup>), *B. septatus* (Gelpke<sup>7</sup>), and *Corynebacterium lymphae vaccinae* (Levy and Fickler<sup>8</sup>). Some investigators consider all these forms as varieties of either *B. pseudodiphthericus* or *B. xerosis*, while others make separate species of some or all. On the other hand, Roux and Yersin<sup>9</sup> and, more recently, Behring<sup>10</sup> look upon the diphtheria group as but a single species, regarding the morphological and biological differences between the types as insufficient to justify establishing new species. Their views, however, have found few adherents.

The important features of typical examples of the three main forms may be summarized as follows:

### MORPHOLOGY.

*B. diphtheriae*: Long, slender rods, straight or slightly bent.

*B. pseudodiphthericus*: Short, plump, straight rods.

*B. xerosis*: Similar to *B. diphtheriae*.

### NEISSER STAIN.

*B. diphtheriae*: Babes-Ernst granules demonstrable.

*B. pseudodiphthericus*: Solid-staining.

*B. xerosis*: Granules, if present, appear late (after 24 hours).

\* Received for publication July 8, 1908.

## LÖFFLER'S INSPISSATED SERUM.

*B. diphtheriae*: Growth is rapid and luxuriant. Vigorous development occurs during the first 12 hours and the maximum is practically reached in 24 hours. Consistency of the growth is firm and coherent and its surface dull and grayish in appearance.

*B. pseudodiphthericus*: Growth is meager. Development during the first 12 hours is slow, and at 24 hours appreciably less than that of the Klebs-Löffler bacillus. The consistency is soft and even viscid. The surface is moist, smooth, lustrous, and pure white.

*B. xerosis*: Growth is very scanty. No development is visible until after 24 hours. The consistency resembles that of *B. diphtheriae*. The surface is dull, gray, and extremely dry.

## AGAR.

*B. diphtheriae*: Growth is slow and meager. Colonies are small, delicate, and transparent with a dull, gray surface.

*B. pseudodiphthericus*: Growth is rapid and luxuriant. Colonies are white, moist, lustrous, and spreading.

*B. xerosis*: Very scanty growth, frequently invisible for two or even three days. Colonies are very small and transparent and present a dull surface.

## PATHOGENICITY.

*B. diphtheriae*: In guinea-pigs causes edema, petechiae, or larger hemorrhages, and a fibrinous exudate at the site of subcutaneous inoculation. The adrenals are enlarged and hemorrhagic and frequently the lymph-glands are likewise. More or less serous exudate is found in the peritoneal cavity and sometimes in the pleural cavities as well. The animal either dies acutely of toxemia or gradually wastes away and dies of cachexia weeks afterward, according to the degree of virulence of the organism. The rabbit is equally susceptible but the lesions are not so constant.

*B. pseudodiphthericus* and *B. xerosis* are non-pathogenic.

## SOLUBLE TOXIN PRODUCTION.

Demonstrable only with *B. diphtheriae*.

## ACID PRODUCTION IN CARBOHYDRATE BROTH.

*B. diphtheriae*: Dextrose (and dextrin).

*B. pseudodiphthericus*: None.

*B. xerosis*: Dextrose (and saccharose).

Apparently the differences exhibited are pronounced enough to warrant acceptance of these three forms, at least, as distinct species. The existence of definite and significant points of difference, however, is but one of the two requisites for this recognition, the other being permanence of the characters, and while the first is established, the latter is not beyond question. This can best be shown, perhaps, by taking each character separately and reviewing chronologically the more salient researches thereon.

*Morphology*.—Zarniko,<sup>11</sup> as early as 1889, reported that he was unable to correlate the pathogenicity with the morphology of diphtheria cultures.

Peters<sup>12</sup> found two types of pathogenic diphtheria bacilli which differed morphologically and remained distinct after two years of cultivation except that the shorter approached the pseudodiphtheria type and became avirulent. Nevertheless he does not regard *B. pseudodiphthericus* as an attenuated *B. diphtheriae*.

Ferré and Creignou<sup>13</sup> and Gouguenheim and Dutertre<sup>14</sup> could establish no relation between morphology and virulence.

Kurth<sup>15</sup> believes that the Klebs-Löffler and the Hofmann-Wellenhof organisms can be distinguished by merely noting the length of the bacilli.

De Simoni<sup>16</sup> found a spore-bearing organism of the pseudodiphtheria morphology in ozena secretion.

Wesbrook, Wilson, and McDaniel<sup>17</sup> described three principal (granular, barred, solid-staining) and some 28 secondary forms of diphtheria bacilli. They report that in pure cultures from typical clinical cases of diphtheria there is never complete uniformity in the shape, size, and staining reactions of the individual bacilli and that even a moderate degree of uniformity is exceptional. It appears probable, therefore, that the metamorphosis of the classical *B. diphtheriae* into the solid-staining pseudodiphtheria type occurs rather readily in the throat and that the converse may occur.

Gorham<sup>18</sup> corroborates the above as to the change of the granular to the solid-staining type during convalescence. He considers this due to the production of immune bodies and that it is accompanied by loss of virulence.

Macé<sup>19</sup> found no relation between morphology and virulence in cultures he examined.

Lesieur<sup>20</sup> found that some bacilli of the pseudodiphtheria type may be highly virulent. By animal passage in collodion sacs through three rabbits he found that Hofmann-Wellenhof forms took on the morphology of *B. diphtheriae*, while on the other hand by cultivation for eight months in diffuse light he changed three diphtheria strains to forms indistinguishable from *B. pseudodiphthericus*.

Ohlmacher<sup>21</sup> similarly changed the morphology of a short, solid form to the slender, curved type and a granular to the short, pseudodiphtheria appearance (though it still formed acid) by means of animal passage.

Denny<sup>22</sup> made a very careful study of the morphology of serum cultures at intervals during the first 12 hours of growth. In the case of 10 Klebs-Löffler strains he found that they first appeared as solid-staining forms which later changed to the granular type and exhibited elongated forms, swollen ends, and tinctorial segmentation. He found that unfavorable conditions of growth and symbiosis with other bacteria generally delayed the development of the granular forms and suggests that symbiosis (e. g., with streptococci) may account for the change to the solid type during convalescence. Thirty-five strains of *B. pseudodiphthericus* always presented the short form, while eight strains of *B. xerosis* passed through the same stages as the Klebs-Löffler, although usually they were shorter and thicker than the latter. He believes, nevertheless, that the three types can be regarded as separate species and distinguished from one another if all the stages of their development are considered.

Hamilton<sup>23</sup> isolated some strains from scarlatina, measles, and diphtheria morphologically identical with the Klebs-Löffler bacilli but which were regarded as *B. pseudodiphthericus* on the ground of serum reactions and of pathogenicity.

Hadley<sup>24</sup> reports transforming the granular into the solid-staining type and *vice-versa* by passage through guinea-pigs.

*Babes-Ernst granules*.—Babes<sup>25</sup> was probably the first to see the characteristic granules in cultures of diphtheria bacilli. He thought them spores at the time.

Ernst<sup>26</sup> about the same time reported seeing the granules in *B. xerosis*.

Cronch,<sup>27</sup> who stained the granules with 1 per cent solution of methyl green, observed that they appeared in all the strains of the group but considerably earlier in the Klebs-Löffler bacillus than in the related types. He regarded this as characteristic of *B. diphtheriae*.

In 1897 M. Neisser<sup>28</sup> published the method, now universally employed, for staining the granules in the diphtheria bacillus. He offered it as a reliable means for distinguishing between the true and the false diphtheria forms, although admitting that the granules were present occasionally in *B. pseudodiphthericus*, though never in *B. xerosis*. They appeared between the ninth and twenty-fourth hours.

Heinersdorff<sup>29</sup> found the granules in *B. diphtheriae* appeared between the eighth and twelfth hours, while those he found in *B. xerosis* were not visible until after 24 hours. He examined 60 strains of *B. xerosis* of less than 12 hours growth but could not find typical granules in any.

Kurth<sup>15</sup> and also Gramakowsky<sup>30</sup> found the granules present in *B. pseudodiphthericus*.

Schanz<sup>31</sup> found the Babes-Ernst granules in *B. xerosis*, although later than in *B. diphtheriae*.

Cobbett<sup>32</sup> reported finding no granules in 69 strains of *B. pseudodiphthericus*, using Neisser's stain.

Lesieur<sup>20</sup> found the granules in 80 per cent of the diphtheria and in 20 per cent of the pseudodiphtheria strains examined. He could ascertain no constancy in the time of their appearance. Prolonged cultivation in diffuse light caused the granules to disappear.

Denny<sup>22</sup> states that in contradistinction to the granules of *B. diphtheriae*, those of *B. xerosis* stain readily with Löffler's methylene blue but poorly with the Neisser granule stain. He found granules present also in *B. pseudodiphthericus*, though but rarely.

Gordon<sup>33</sup> observed the granules in *B. coryzae segmentosus* and in four other diphtheroids.

Graham-Smith<sup>34</sup> was able to demonstrate these structures in a considerable number of diphtheroid "species."

*Pathogenicity*.—Löffler<sup>35</sup> noted that *B. diphtheriae* in cultures loses its virulence after a time without any concomitant change in cultural characters.

Roux and Yersin,<sup>9</sup> by making throat cultures at various stages after the termination of the disease, discovered that the bacilli in the mouth gradually became attenuated. Virulent diphtheria bacilli, cultivated at 39.5° C. or in a current of air, became attenuated and approached the characters of *B. pseudodiphthericus*, while Klebs-Löffler bacilli of low virulence could be made highly pathogenic by passage through a guinea-pig, in symbiosis with the streptococcus of erysipelas. The experiment did not succeed, however, when totally avirulent bacilli were used.

Escherich,<sup>36</sup> Goldscheider,<sup>37</sup> Sudeck,<sup>38</sup> and Zupnik<sup>39</sup> were unable to confer virulence on strains of *B. pseudodiphthericus*.

Kruse and Pasquale<sup>6</sup> cultivated a diphtheroid form, which they call *B. clavatus*, from a liver abscess in amebic dysentery. It was, however, non-pathogenic to animals.



Bernheim<sup>40</sup> reported that wholly avirulent Klebs-Löffler bacilli became virulent if cultivated on streptococcus filtrates, while the Hofmann-Wellenhof bacillus did not.

Bardach<sup>41</sup> raised the virulence of slightly pathogenic diphtheria bacilli by passage through the dog.

Trumpp<sup>42</sup> converted an avirulent *B. pseudodiphthericus* (it produced acid, however) into a typically virulent form by passage through guinea-pigs simultaneously with sub-lethal doses of diphtheria toxin.

Spronck<sup>43</sup> observed that *B. pseudodiphthericus* and *B. xerosis* produced slight local lesions, such as induration and edema, at the point of inoculation which were similar to those produced by true diphtheria bacilli of low virulence.

Hewlett and Knight<sup>44</sup> changed a typically virulent *B. diphtheriae* into a non-virulent organism of the pseudodiphtheria type by 17 hours exposure to a temperature of 54° C. Subsequent attempts to repeat this experiment were unsuccessful. They also report changing a *B. pseudodiphthericus* strain into a form typically virulent by cultivation and by passage through guinea-pigs.

Shattock<sup>45</sup> investigated the possibility of increasing the virulence of *B. diphtheriae* by means of sewer air. He found that two months cultivation of a slightly virulent Klebs-Löffler bacillus in a current of sewer air did not exalt its pathogenicity.

Martin<sup>46</sup> raised the virulence of feebly pathogenic diphtheria bacilli by cultivation in collodion sacs in the peritoneal cavity of rabbits.

Bergey<sup>47</sup> was unable to give virulence to avirulent pseudodiphtheria forms.

Davis<sup>48</sup> described 12 diphtheroid strains, isolated from cases of scarlatinal otitis media, which were pathogenic to guinea-pigs on subcutaneous inoculation, producing peritonitis and septicemia. Antitoxin afforded no protection against any of them. Williams<sup>49</sup> simultaneously reported finding the same organism.

Richmond and Salter<sup>50</sup> and Salter<sup>51</sup> changed five pseudodiphtheria bacilli into forms resembling the typical *B. diphtheriae* and specifically virulent for guinea-pigs, by passage through gold-finches. The organism now formed acid in broth and its action was neutralized by diphtheria antitoxin.

De Simoni<sup>52</sup> reported success in making pseudodiphtheria bacilli virulent by growing them on the surface of old tetanus cultures.

Levy and Fickler<sup>5</sup> described a diphtheroid which they have named *Corynebacterium lymphae vaccinalis*. While producing no soluble toxin, it causes abscess and chronic suppuration in animals.

Neumann<sup>53</sup> obtained an "hereditary" attenuation of the Klebs-Löffler bacillus by the prolonged action of increased atmospheric pressure.

Martin,<sup>46</sup> Spronck,<sup>54</sup> Park and Williams,<sup>55</sup> Nicolle,<sup>56</sup> and Nicolas and Arloing<sup>57</sup> have exalted the virulence of this species by means of special cultural media to favor stronger toxin production.

Levy<sup>58</sup> repeated Roux and Yersin's work in increasing virulence by animal passage in symbiosis with streptococci. He was unable to make pseudodiphtheria strains virulent.

Lesieur<sup>20</sup> found that of 30 strains of pseudodiphtheria bacilli, 13 produced appreciable quantities of toxin, and 10 more formed traces of it. Of 40 strains of Klebs-Löffler bacilli, 12 produced toxin in only minute quantities and three produced none whatever. He succeeded in causing diphtheria in guinea-pigs with Hofmann-Wellenhof bacilli both by inoculation with large quantities of unchanged bacilli and by raising the virulence of the latter by collodion sac animal passage. The morphol-

ogy changed at the same time as the virulence. Virulent Klebs-Löffler forms were made to lose their pathogenicity by long exposure to diffuse light. This has also been accompanied, as noted above, by a transformation to the pseudodiphtheria morphology. Further, he was able to make two out of four strains of *B. pseudodiphthericus* virulent by repeated transfers in broth and one out of three by a single passage through broth in symbiosis with *Staph. pyogenes aureus*.

Ohlmacher<sup>21</sup> found strains of *B. pseudodiphthericus* which were virulent to guinea-pigs. He also exalted the virulence of bacilli of the short, solid-staining type by passage through a guinea-pig.

Cobbett<sup>32</sup> investigated the organisms secured in an epidemic at Cambridge. He was able to find no intermediate degrees of virulence among the Klebs-Löffler bacilli and none of the Hofmann-Wellenhof type were virulent.

Williams<sup>50</sup> denies that Klebs-Löffler bacilli in the throats of diphtheria patients change to the pseudodiphtheria type, and states that they do not alter when transferred to other throats. Moreover, only a slight decrease in virulence results from prolonged cultivation of *B. diphtheriae* on artificial media. The experiments of increasing the virulence in non-pathogenic strains by symbiosis with streptococci and by passage through gold-finches were repeated but could not be confirmed.

Ruediger<sup>60</sup> described pseudodiphtheria bacilli obtained from scarlatinal throats which were virulent to guinea-pigs.

Graham-Smith<sup>61</sup> found an organism resembling that described by Davis, in a diphtheria epidemic.

Hamilton<sup>23</sup> collected some 15 diphtheria strains pathogenic to guinea-pigs but which did not produce the lesions of the Klebs-Löffler bacillus in them, nor produce soluble toxin. Antitoxin did not protect guinea-pigs against them.

Graham-Smith<sup>34</sup> obtained results similar to those of Cobbett, that is, he found no partially attenuated Klebs-Löffler, nor any virulent Hofmann-Wellenhof bacilli.

Zinsser<sup>62</sup> studying some 79 diphtheria strains found no virulent pseudodiphtheria or xerosis bacilli, but two diphtheroids had some slight pathogenicity, and one of them gave some immunity against *B. diphtheriae*.

*Immune serum reactions.*—De Martini<sup>63</sup> reported that pseudodiphtheria bacilli grow freely in antitoxin while *B. diphtheriae*, whether virulent or avirulent, does not develop. This interesting observation has never been confirmed.

Spronck,<sup>43</sup> in 1896, described a method, which is now known as "Spronck's Test," for distinguishing between the Klebs-Löffler bacillus and *B. pseudodiphthericus* and *B. xerosis*. He injected guinea-pigs with diphtheria antitoxin and then inoculated them with the culture to be examined. If the latter happened to be true diphtheria bacilli the animal showed complete protection, if not, local infiltration and edema was produced as in the controls. Of course, the test is not applicable to wholly avirulent strains. Spronck's results were soon confirmed by several investigators including C. Fränkel<sup>64</sup> who was so impressed with their significance that he was lead to retract his acceptance of Roux and Yersin's theory of the biologic identity of the members of the diphtheria group.

Glücksman<sup>65</sup> showed that animals immunized with *B. pseudodiphthericus* are not protected against *B. diphtheriae*.

Bergey<sup>46</sup> could produce no immunity against virulent by means of non-virulent pseudodiphtheria bacilli.

Salter<sup>51</sup> reported that he had found in filtrates of *B. pseudodiphthericus* substances identical with the toxoid produced by the Klebs-Löffler bacillus.

Nicolas<sup>66</sup> discovered that antidiphtheritic serum agglutinated the Klebs-Löffler bacillus, thus offering a simple and excellent means of diagnosis. On repeating the experiments, however, Nicolle<sup>67</sup> was unable to confirm his results. Nicolas,<sup>68</sup> on going more deeply into the matter, found that the agglutinability of *B. diphtheriae* is not constant and that the differences which had been observed were due to differences in the races of bacilli used. He stated that no relation between virulence and agglutinability appeared to exist. His results are confirmed in general by Bruno.<sup>69</sup>

Lubowski<sup>70</sup> also employed agglutination experiments to separate the true from the false diphtheria bacillus. He immunized goats with avirulent Klebs-Löffler bacilli and secured a serum which agglutinated 23 races of *B. diphtheriae* but had no action on *B. pseudodiphthericus*. On the other hand, Lesieur<sup>71</sup> found there was no difference in behavior toward a specific serum *in vitro* between the true diphtheria and the pseudodiphtheria bacilli. He also states that *B. pseudodiphthericus* produces a body similar to, if not identical with, the toxone of *B. diphtheriae*.

Gordon<sup>33</sup> found that there were great variations in the results of agglutination experiments even with Klebs-Löffler strains, thus confirming the observations of Nicolas. He thinks this depends on whether or not the bacilli used as antigens were recently isolated.

Schwoner<sup>72</sup> thinks reliable results are obtainable in differentiating *B. diphtheriae* from *B. pseudodiphthericus* by means of agglutination in specific sera.

Lambotte<sup>73</sup> found evidence that the amboceptors of *B. diphtheriae* and *B. pseudodiphthericus* are not identical.

Cobbett<sup>74</sup> and likewise Hewlett<sup>75</sup> were unable to confirm the results of Salter in regard to toxoid production. The same conclusion was reached by Petrie<sup>76</sup> for he found that filtered cultures of pseudodiphtheria bacilli do not immunize horses so that diphtheria antitoxin is produced.

Hamilton and Horton<sup>77</sup> reported that the antibodies formed on immunization of goats and rabbits with virulent pseudodiphtheria bacilli are distinct from those produced by avirulent pseudodiphtheria forms as well as those of *B. diphtheriae*.

*Cultural characters.*—Abbott<sup>78</sup> found an organism indistinguishable from the non-pathogenic *B. diphtheriae* except in that it formed a "dirty brown layer" on potato.

McClure<sup>79</sup> and also Gordon<sup>25</sup> have described diphtheria-like bacilli which strongly acidified and coagulated milk and Klein<sup>5</sup> isolated a similar organism, *B. diphtheroides*, which not only strongly acidified and coagulated milk, but also liquefied Löffler's inspissated serum and stained with difficulty with the ordinary anilin dyes.

Zupnik<sup>39</sup> found two distinct strains existing symbiotically in the same culture of *B. diphtheriae*. They differed in regard to appearance in broth, vigor of growth, the morphology of the colonies, Gram stain, and pathogenicity.

Graham-Smith<sup>34</sup> uses as one means of differentiating between the Klebs-Löffler and the Hofmann-Wellenhof types the character of their growth on his potato-agar medium. He described a number of diphtheroids secured from different parts of the body some of which actually coagulate milk, produce indol, reduce nitrates, liquefy Löffler's serum and gelatin, and form a yellow growth on potato.

Schwoner<sup>80</sup> showed that about 70 per cent of the true diphtheria strains are able to cause hemolysis in rabbits' corpuscles (this has been observed by others also), while *B. pseudodiphthericus* (typical) failed to produce hemolysis.

*Löffler's serum and agar.*—Regarding the growth of the members of the group on these media, it is unnecessary to take time for a rehearsal of the literature, as everyone who has worked with diphtheria bacilli knows how extremely variable they are in this respect.

*Acid fermentation of carbohydrates (and alcohols).*—To facilitate comparison, as well as to save time, I have tabulated the results of the important investigations on this point. In most cases 1 per cent of the sugar was used, usually dissolved in broth, but in some cases in the serum-water medium of Hiss<sup>81</sup>. In all cases the production of acid was recognized by means of indicators in the medium.

TABLE 1.

Author	Dextrose	Levulose	Galactose	Maltose	Saccharose	Dextrin	Lactose	Mannite	Glycerin	Arabinose	Raffinose	Dulcite	Glycogen	Erythrite	Starch	
T. Smith <sup>72</sup> .....	+				—		+									B. diphtheriae
Blumenthal <sup>84</sup> .....	+						+									
Axenfeld <sup>84</sup> .....	+															
Frankel <sup>85</sup> .....	+	+	+	—	+		—	—	+	—	—	—	—	—	—	
Martin <sup>10</sup> .....	+															
Bronstein and Grünblatt <sup>86</sup> .....	+															
Knapp <sup>87</sup> .....	+			+	—	+	—	+								
Hamilton and Horton <sup>77</sup> .....	+	+	+	+	—	+	±	±	+							
Graham-Smith <sup>88</sup> .....	+	+	+	±	—	+	±	±								
Rothe <sup>89</sup> .....	+	+	+	+	—	+	—	—				—				
Zinsser <sup>62</sup> .....	+	+	+	+	—		+	—								B. pseudodiphthericus
Arkwright <sup>90</sup> .....	+	+	+	+	—		+	—								
Eyre <sup>91</sup> .....	x						x									
Peters <sup>92</sup> .....	x						+									
Axenfeld <sup>84</sup> .....	x															
Frankel <sup>85</sup> .....	—															
Lawson <sup>93</sup> .....	—															
Gordon <sup>13</sup> .....	—															
Bronstein and Grünblatt <sup>86</sup> .....	—															
Denny <sup>22</sup> .....	x															
Knapp <sup>87</sup> .....	—			—	—	—	—	—								B. xerosis
Hamilton and Horton <sup>77</sup> .....	—				—	—	—	—								
Graham-Smith <sup>88</sup> .....	—	—	—	—	—	—	—	—	—							
Benham <sup>24</sup> .....	—	±	—	—	±	—	—	—								
Rothe <sup>89</sup> .....	—	±	—	—	—	—	—	—				—				
Zinsser <sup>62</sup> .....	—	—	—	—	—	—	—	—								
Arkwright <sup>90</sup> .....	—	—	—	—	—	—	—	—								
Eyre <sup>91</sup> .....	—															B. cor yzae segment osus
Axenfeld <sup>84</sup> .....	—															
Frankel <sup>85</sup> .....	x															
Denny <sup>22</sup> .....	+															
Knapp <sup>87</sup> .....	+			+	+	—	—	+								
Graham-Smith <sup>88</sup> .....	+	+	—	—	+	—	—		+							
Benham <sup>24</sup> .....	+			—	—	—	—	—								
Rothe <sup>89</sup> .....	—	—		—	—	—	—	—								
Zinsser <sup>62</sup> .....	+	+	+	+	+	—	—	—				—				
Gordon <sup>13</sup> .....	—															
Gordon <sup>13</sup> .....	+			—	—	—	—	—	—							Diphtheroids
Graham-Smith <sup>88</sup> .....	+	+	+	+	+	—	+	—	—							
Benham <sup>24</sup> .....	+	+	—	—	—	—	—	—	—							
Arkwright <sup>90</sup> .....	+	+	—	—	—	—	—	—	—							
Graham-Smith <sup>88</sup> .....	±	±	±	±	—	±	—	±	—			—				
Zinsser <sup>62</sup> .....	+	+	+	+	—	±	—	±	—							
Arkwright <sup>90</sup> .....	+	+	+	+	—	±	—	±	—							
Graham-Smith <sup>88</sup> .....	±	±	±	±	—	±	—	±	—							
Zinsser <sup>62</sup> .....	+	+	+	+	—	±	—	±	—							
Arkwright <sup>90</sup> .....	+	+	+	+	—	±	—	±	—							

NOTE.—Kurth<sup>15</sup> has described a *B. pseudodiphthericus acidum faciens* which forms as much acid in dextrose as the Klebs-Löffler bacillus.

+ = acid production.

— = alkali production.

x = no change.

The foregoing review requires no discussion to convince one that aside from the immunity and fermentation reactions the means of differentiating accurately between the members of this group are valueless. As for the serum reactions, however, suffice it to say that as yet it is unknown whether specific antigenic capacity is a species or merely a racial character, indeed, even the work on this subject in connection with the diphtheria group, as cited above, contains much that strongly hints at the latter interpretation. Until this is disproved, then, evidence that the diphtheria group comprises more than one species, apparently must be deduced from fermentative characters alone. A glance at the preceding tabulation shows that there are many discrepancies in the results reported, but more careful perusal almost satisfies one that certain of the reactions stand out as being comparatively undisputed. Compiling these presumably valid reactions, it is found that they form a scheme whereby the three important members of the group might be sharply differentiated, thus:

TABLE 2.

	Dextrose	Dextrin	Saccharose
<i>B. diphtheriae</i> .....	+	+	—
<i>B. pseudodiphthericus</i> .....	—	—	—
<i>B. xerosis</i> .....	+	—	+

Now, it is quite readily conceivable that these alone may be the essential diagnostic species characters, and in that case the lack of uniformity in behavior toward the other sugars is of no consequence. Inasmuch, however, as the propriety of dividing the diphtheria group into distinct species appears to rest exclusively upon these few reactions, it seems highly important to settle their validity as species characters. To justify their use as species characters these fermentative powers must fulfil two requirements. In the first place, they must be permanent and hence unalterable in spite of prolonged artificial or natural selection so that organisms of the species from whatsoever source will have practically the same zymogenic characters. In the second place, there must be no intermediary forms, for this would indicate either that members of the species could gradually lose these characters or else that the latter could be acquired by another species. If, therefore, one or both of these conditions can be demon-

strated as lacking in this case, we must relinquish the fermentation test.

The following study was undertaken for that purpose, and also, incidentally, to determine how far such a fundamental biologic character as enzyme production can be altered artificially.

The problem was approached in two ways. The first was to secure as many strains of this group as possible and determine by titration the exact amount of acid produced in the different sugars, with the object of learning to how great a degree the fermentative powers of these bacteria vary in nature. If it were to transpire that the organism could be grouped into two or more definite aggregations on the basis of the extent of their zymogenic power, the presumption would be that this was constant, while, on the other hand, if it were found that such grouping was not possible because of the occurrence of intermediate or transitional forms, this would necessarily have to be regarded as evidence of the instability of the characters and of the likelihood that the latter can be assumed by other types in the group.

For this purpose 103 strains of the diphtheria group were secured, some from the laboratories of other universities, but most were isolated from smears on Löffler's medium sent to the Chicago City Health Department for diagnosis.\* Immediately after isolation the organisms were inoculated into tubes containing 1 per cent of dextrose, dextrin, maltose, or saccharose in sugar-free infusion broth, incubated at 37° C. for three days, and the amount of acidity produced was determined by titration with N/20 NaOH. The object of titrating for the exact amount of acid instead of merely demonstrating the presence of acid by adding an indicator to the medium as others have done, was that in this way can be detected the existence of intermediate forms, the crucial point in the whole question, while by the older method this is impossible. By the latter can be learned whether a given bacterium does or does not produce acid but no information is given regarding the extent to which acid is produced. This can be obtained only by means of titration. In other words, the indicator method emphasizes uniformity and obscures the variations, while the titration method emphasizes the variations.

\* I am indebted to Dr. Weber of the Health Department Laboratory for the smears and I take this opportunity to thank him.

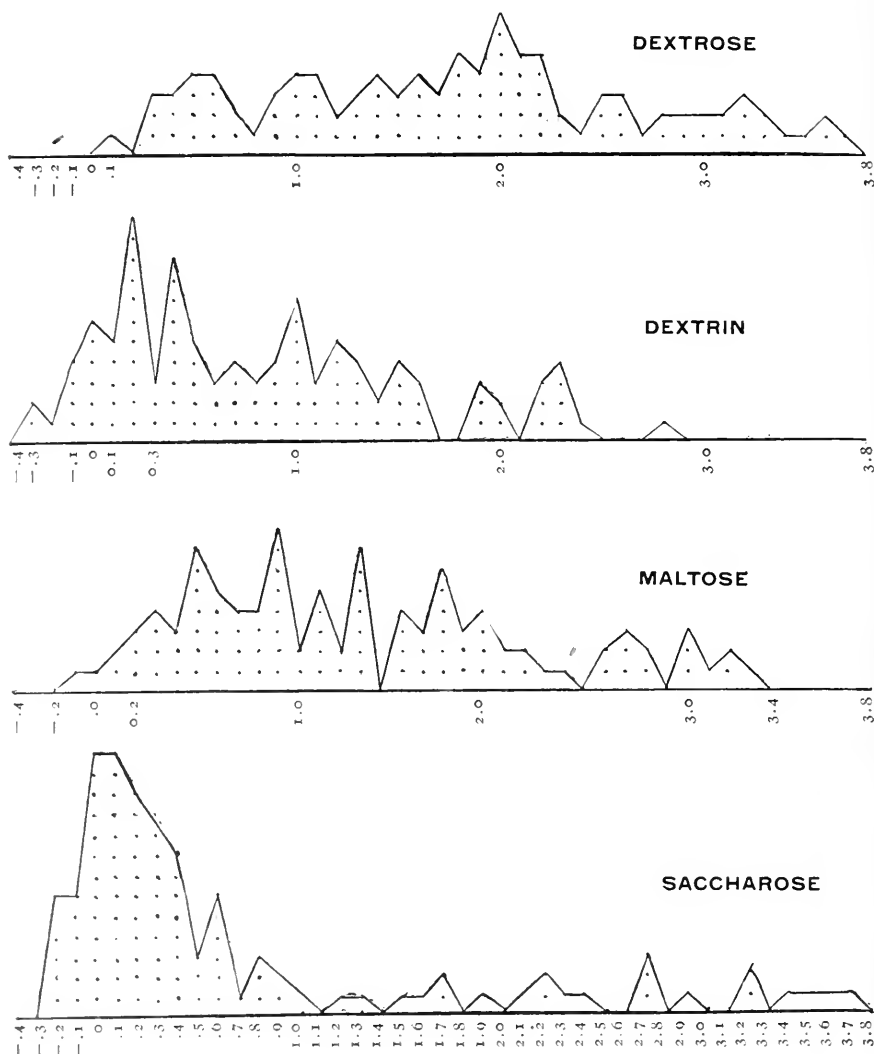
TABLE 3.

No.	Strain	Dex-trose	Dex-trin	Mal-tose	Sac'a-rose	No.	Strain	Dex-trose	Dex-trin	Mal-tose	Sac'a-rose
1	15-	4.0	2.3	3.1	2.1	53	120	1.8	1.5	1.3	0.0
2	Herdman	3.7	0.5	0.6	3.7	54	410	1.8	2.3	2.7	2.2
3	683C	3.6	1.2	3.0	3.2	55	Buchanan-	1.8	0.4	0.7	0.2
4	Hudson	3.6	-0.2	1.0	3.5	56	Minn. D	1.7	1.2	0.9	0.3
5	72	3.5	1.2	0.9	2.4	57	155	1.7	0.9	2.2	0.8
6	477-	3.4	1.6	3.3	2.3	58	874	1.7	2.4	1.8	0.4
7	308-	3.3	1.5	1.3	0.0	59	58A	1.6	1.0	1.1	0.3
8	329-	3.3	2.2	1.4	-0.2	60	167	1.6	1.0	1.8	0.6
9	114	3.2	0.25	2.8	-0.2	61	594	1.6	1.0	1.7	0.1
10	Granger	3.2	0.4	0.5	3.6	62	715	1.6	0.7	0.8	0.5
11	65	3.2	-0.3	0.4	3.4	63	338	1.5	0.7	1.7	0.35
12	277	3.1	-0.3	0.5	3.2	64	682	1.5	0.5	1.3	1.7
13	442-	3.1	0.3	0.9	2.9	65	Bapt-	1.5	0.1	1.9	0.0
14	19-	3.0	0.4	3.0	0.6	66	27A	1.4	0.1	1.6	1.5
15	148-	3.0	1.9	3.2	2.7	67	74-	1.4	0.9	0.8	2.1
16	85	2.9	2.0	2.4	0.4	68	138	1.4	1.0	1.8	0.1
17	633	2.9	2.3	3.2	0.0	69	776A	1.4	0.0	1.3	0.0
18	23D-	2.8	0.5	1.8	2.7	70	M. I.-	1.3	0.9	1.1	0.0
19	520	2.8	1.3	2.7	0.0	71	101	1.3	1.1	1.2	0.4
20	342	2.7	1.4	2.0	0.6	72	792	1.3	0.5	0.6	0.5
21	8-	2.6	1.0	1.6	0.2	73	32	1.2	0.2	0.9	1.0
22	Davis-	2.6	0.5	1.3	0.2	74	572	1.2	2.2	1.0	-0.1
23	401A	2.6	1.5	2.7	0.3	75	13-	1.1	0.0	2.2	0.1
24	23E-	2.5	0.2	1.4	2.7	76	388	1.1	0.1	0.8	1.6
25	370	2.5	1.9	2.3	0.2	77	441-	1.1	0.7	1.1	0.4
26	528	2.5	0.0	1.9	1.9	78	K	1.1	0.0	1.3	0.8
27	739	2.4	1.5	2.0	0.2	79	Park-	1.0	0.4	0.6	0.05
28	20B	2.3	1.3	1.3	0.9	80	252	1.0	0.7	1.1	0.3
29	214-	2.3	1.1	2.0	0.4	81	484-	1.0	0.4	0.7	0.4
30	70-	2.2	1.35	3.0	0.0	82	Brown P. V-	1.0	0.2	0.8	0.2
31	4-	2.2	0.8	2.1	0.8	83	222	0.9	0.4	1.2	0.3
32	46	2.2	0.4	2.1	-0.05	84	440-	0.9	0.6	0.9	0.6
33	134	2.2	1.9	1.9	0.3	85	W	0.9	0.2	0.2	1.2
34	848	2.2	0.8	2.6	0.1	86	386B	0.8	0.2	0.4	0.1
35	45C	2.1	0.2	0.9	1.7	87	220	0.7	-0.1	0.4	-0.2
36	115A	2.1	2.0	2.8	0.2	88	Calhoun	0.7	0.0	0.0	0.1
37	211	2.1	0.8	-0.1	0.7	89	Minn. P.-	0.6	0.2	0.6	-0.2
38	239A	2.1	1.6	0.4	0.9	90	G	0.6	0.0	0.5	0.1
39	551	2.1	2.3	1.7	0.15	91	Disp.	0.6	-0.1	0.5	-0.2
40	Ch. P.	2.0	1.1	0.5	0.6	92	Simpson-	0.6	1.0	0.5	0.1
41	44	2.0	2.8	0.9	0.6	93	Harv. P.-	0.5	0.2	0.6	-0.1
42	Kyes	2.0	1.3	2.2	0.1	94	39	0.5	0.3	0.7	0.0
43	73B	2.0	0.6	1.1	0.5	95	V	0.5	0.2	0.2	0.4
44	284-	2.0	0.0	1.6	-0.1	96	Gage-	0.5	0.4	0.7	0.3
45	548	2.0	2.2	2.6	0.0	97	335	0.4	0.1	0.1	0.15
46	967	2.0	1.6	1.8	1.3	98	659	0.4	-0.1	0.3	0.1
47	78	1.9	0.6	0.9	0.2	99	811	0.4	-0.1	0.3	0.0
48	103-	1.9	1.0	1.4	-0.1	100	89	0.3	0.1	0.5	-0.2
49	516	1.9	1.2	1.8	0.0	101	431-	0.3	0.0	0.0	0.1
50	683A	1.9	0.4	0.2	0.0	102	Brown P. VI-	0.3	0.2	0.3	0.0
51	16B	1.8	1.2	1.6	-0.05	103	Brown P. 31-	0.1	0.2	0.3	0.1
52	50	1.8	1.4	1.9	0.2						

The results of the experiment, which are shown in Table 3 arranged in the order of the dextrose-fermenting power of the strains, demonstrate that the forms cannot be separated on the basis of their fermentations into definite groups. This is shown more clearly when these data are plotted in frequency curves. Inspection of these (Chart 1) reveals the fact that in the case of none of the four carbohydrates is it possible to make a sharp line of division at any point. It must be admitted that the results of previous investigations would cause one to expect that the curves, even if continuous, would reveal

several "peaks" separated by distinct "valleys" to account for the rather clean-cut results of the indicator method experiments, but a moment's reflection will explain the apparent discrepancy for with an indicator the most level titration "plateau" may be divided into two sharply separated groups.

CHART 1.



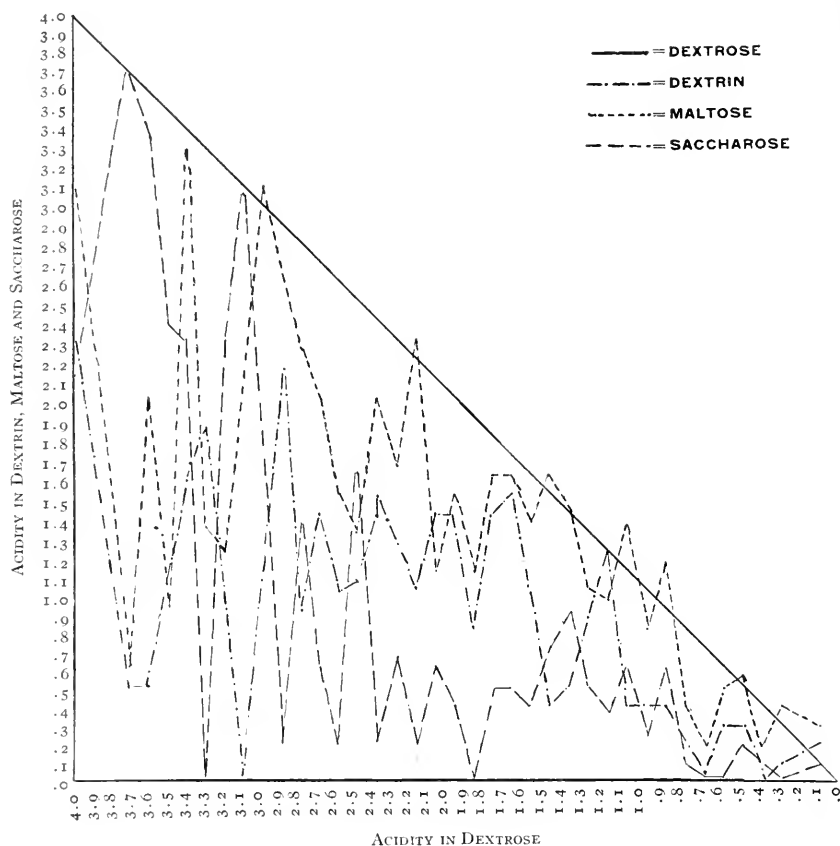


The curves, although of the same general significance, show minor differences among themselves that are of sufficient importance to merit mention. In the first place, the type of each curve is different. The extremes are dextrose and saccharose, the former showing a remarkably evenly graded distribution of zymogenic power over an extremely wide range (almost 4 per cent) while the latter, although really covering an even more extensive range, is for the most part limited to a variation of only about 1 per cent and so situated as to demonstrate that the majority of the members of the diphtheria group possess rather small power to ferment saccharose. The curves of dextrin and maltose are intermediate between the other two, the former resembling saccharose more closely and the latter dextrose. In the second place, there does not appear to be any relation between high fermentative power with one sugar and the ability to break up other carbohydrates, that is, a bacterium, whose fermentative powers in respect to one sugar are exaggerated, may have a subnormal capacity in regard to other sugars and *vice-versa*, although when the fermenting capacity for dextrose is low that for the others is correspondingly depressed. The point is presented graphically in Chart 2 where the dextrin, maltose, and saccharose fermentations are plotted in relation to the dextrose.

The next experience was to determine the limits of the variability in the zymogenic power. Its bearing on the general problem is that if prolonged artificial selection should prove unable to change materially the fermentative character of one of these organisms, strong evidence of the permanence of that character would be afforded. On the other hand, if selection can materially alter the power, exalt or depress it at the will of the experimenter, it is but reasonable to expect similar changes in nature and thus would be explained the occurrence of almost every degree of development of the character in different strains, as shown in Table 3. In fact, such an alteration would be reasonably conclusive proof that the fermentative properties of the members of this group cannot be utilized as a differentiating species character. Such actually proved to be the case in this experiment.

A culture of the Klebs-Löffler bacillus, isolated from a clinical case by Professor Preston Kyes of the Pathology Department, was plated, one colony isolated (the descendant of a single cell presumably), and

CHART 2.



inoculated into a series of tubes of sugar-free infusion broth containing 1 per cent of dextrose. After three days of incubation the reaction in each tube was determined by titration, the tube containing the maximum acidity and that with the minimum were plated, and the organisms recovered taken as the High and the Low strains respectively. Each was carried through 36 additional transfers in the same manner, that with the maximum acidity being the only organism saved and carried on in the High strain, and similarly that with the minimum acidity in the Low series. The tubes were incubated for from two to four days before titration, according to convenience, but lack of constancy in this particular cannot be a factor in the results obtained since

the corresponding High and Low strains were always run through together, and it is not the actual amount of acidity produced that is the important element in this case but the difference between that formed by the maximum of one and the minimum of the other strain. To guard against contamination, the strains were usually plated weekly. It was not felt necessary to do this after each transfer for certainly it does not hasten the process of adaptation, on the contrary, if one happens to pick out a colony belonging to the minority of the less adaptable bacilli, results are retarded, at least in the High strains. It seems reasonable to hold that the most adaptable variant in each culture will multiply most rapidly so that when inoculated from tube to tube by means of the platinum loop an excess of this variant is carried over to begin with and more rapid results will be obtained than by inoculation with a colony possibly consisting of the least adaptable variant in the culture. As a matter of fact, indeed, it was not infrequently noticed in the Low series that the culture following plating gave an appreciably smaller amount of acid than did those immediately preceding with which direct inoculation was practiced. Another factor, of less importance, is that direct inoculation saves at least 24 hours in time on each series.

The results of the titrations\* in this experiment are shown in Table 4, and the curves of the acid production by the maximum variant in the High and the minimum variant in the Low strains of

TABLE 4.

The bold-face figures are those of the cultures carried over for the inoculation of the following series. Roman numerals indicate series. H=High strain, L=Low strain.

ORIGINAL	I		II		III		IV		V		VI		VII		VIII	
	H	L	H	L	H	L	H	L	H	L	H	L	H	L	H	L
2.3	2.1	2.0	1.8	1.9	1.9	1.7	1.0	1.6	2.5	1.6	1.9	1.9	2.3	1.6	2.2	<b>1.7</b>
2.2	2.2	1.5	1.6	2.0	1.7	1.6	1.6	1.8	2.4	2.1	1.9	1.8	2.0	2.0	2.0	1.9
2.2	<b>2.4</b>	1.9	1.9	1.7	1.9	1.6	1.5	1.8	<b>2.6</b>	2.0	1.8	1.9	2.3	2.2	2.4	1.9
2.0	1.7	1.7	<b>2.0</b>	2.0	2.0	1.5	1.5	1.6	2.4	1.8	2.0	2.1	<b>2.4</b>	2.2	<b>2.5</b>	2.3
2.1	1.6	2.0	1.7	1.8	2.0	1.7	1.3	1.7	2.1	1.8	2.2	2.0	1.9	1.6	2.3	2.3
2.1	1.7	<b>1.7</b>	1.6	1.7	1.8	1.6	1.4	1.9	2.1	<b>1.4</b>	2.0	2.0	2.2	1.7	2.3	2.0
<b>2.3</b>	1.6	2.3	1.9	1.7	2.0	1.5	1.3	1.5	2.3	1.7	2.1	1.9	2.2	1.6	2.3	1.8
<b>1.9</b>	1.7	2.1	1.5	1.9	1.5	1.6	1.3	<b>1.5</b>	2.3	2.1	1.9	2.1	2.0	2.2	2.1	1.8
1.9	1.9	1.7	1.6	2.1	<b>2.1</b>	1.6	1.4	1.9	2.2	1.7	<b>2.2</b>	1.8	2.0	2.0	2.2	2.1
2.3	1.9	2.1	1.6	1.8	2.1	1.7	1.4	1.6	2.0	1.7	2.0	2.1	2.3	<b>1.4</b>	2.1	1.8
2.0	1.9	1.7	1.8	1.8	2.0	<b>1.5</b>	1.5	2.0	2.4	2.1	1.9	1.9	2.1	1.8	2.4	2.0
1.9	2.0	1.9	1.6	<b>1.6</b>	2.1	1.5	1.4	1.6	2.1	1.6	1.8	1.9	2.0	1.7	2.4	1.7
1.9	1.7	1.7	1.8	1.9	1.8	1.6	<b>1.7</b>	1.7	2.1	1.6	2.1	2.0	1.9	1.6	2.2	2.0
2.0	1.6	1.9	1.8	1.9	2.0	1.5	1.0	1.5	2.1	1.8	2.1	<b>1.8</b>	1.9	1.5	2.0	1.8
2.1	2.0	1.7	1.8	2.0	2.0	1.8	1.4	1.8	2.4	2.1	2.0	2.0	2.2	1.6	2.3	2.1

\* The titrations were performed for me by Miss Ethel Lowenthal, a student in the laboratory.

TABLE 4.—Continued.

IX		X		XI		XII		XIII		XIV		XV	
H	L	H	L	H	L	H	L	H	L	H	L	H	L
2.8	1.7	<b>3.1</b>	2.1	3.3	2.4	2.9	2.3	<b>3.1</b>	2.0	2.4	2.0	3.2	<b>1.5</b>
2.7	2.4	2.0	2.2	3.3	2.2	2.6	2.5	2.7	2.6	2.2	2.6	3.0	2.3
3.1	1.2	2.5	2.6	3.0	2.5	2.7	2.5	2.7	2.0	2.7	2.0	3.3	2.3
3.0	1.0	3.1	2.4	2.6	2.8	<b>3.4</b>	1.9	2.5	2.0	<b>3.1</b>	2.0	2.7	2.1
2.0	1.0	2.7	2.0	3.1	2.3	3.1	2.3	3.0	1.5	2.7	1.5	2.8	2.2
<b>3.2</b>	2.2	2.8	1.9	<b>3.4</b>	2.8	3.2	2.7	2.9	2.3	3.0	2.3	<b>3.4</b>	1.7
3.0	1.8	3.0	2.1	2.6	2.3	3.4	<b>1.8</b>	2.7	2.2	2.9	<b>1.4</b>	3.0	2.0
2.7	<b>1.2</b>	3.0	2.4	2.9	2.4	2.6	2.0	2.4	1.7	2.8	1.7	3.0	1.6
3.1	1.9	2.7	<b>1.6</b>	3.3	2.6	2.7	2.3	2.8	2.0	3.0	2.0	2.9	1.6
3.1	1.4	2.9	2.3	3.3	2.7	2.5	2.2	2.8	2.5	2.6	1.5	2.5	1.8
2.9	1.4	3.0	2.3	2.5	<b>2.2</b>	3.0	2.5	3.0	1.9	2.9	1.9	3.0	2.1
2.8	1.5	2.8	1.9	2.9	2.4	3.1	2.1	2.6	<b>1.4</b>	2.7	1.8	3.1	1.6
3.0	1.0	2.8	2.1	3.2	2.5	2.8	2.1	2.9	1.8				
3.0	1.7	2.6	2.1	3.0	2.7	3.0	2.4	<b>3.2</b>	2.0				
2.9	1.6	2.6	2.2	3.1	2.3	2.7	2.4	3.0	2.1				

XVI		XVII		XVIII		XIX		XX		XXI		XXII	
H	L	H	L	H	L	H	L	H	L	H	L	H	L
3.1	1.7	2.8	1.1	2.9	2.1	3.5	0.7	4.2	0.8	4.2	0.8	3.0	0.1
2.4	<b>1.4</b>	2.5	1.2	2.8	1.4	3.6	<b>0.2</b>	4.4	0.8	3.7	0.6	4.1	-0.1
3.1	1.7	3.1	0.9	3.1	1.6	3.0	0.7	4.1	<b>0.3</b>	3.3	0.7	4.1	-0.1
2.8	2.1	<b>3.2</b>	1.4	<b>3.3</b>	2.0	3.3	0.7	4.1	0.8	4.1	1.1	4.0	-0.1
2.8	2.0	3.0	1.8	3.0	2.2	3.3	1.0	4.5	0.7	3.7	<b>0.6</b>	3.9	0.0
3.0	1.5	2.7	1.7	3.0	1.6	3.6	0.9	4.0	0.7	3.0	0.7	4.0	-0.1
2.9	1.4	2.9	1.2	2.7	1.7	3.3	0.7	3.8	0.8	3.6	0.8	4.1	-0.2
3.0	1.8	3.0	1.2	2.8	1.4	3.4	0.8	4.1	1.0	3.8	1.0	4.0	-0.1
3.0	1.6	2.9	<b>0.6</b>	2.5	2.1	<b>3.9</b>	0.6	4.5	0.7	<b>4.2</b>	0.7	4.1	0.01
<b>3.2</b>	1.8	2.7	1.6	3.1	1.8	3.4	0.6	4.5	0.5	3.4	0.9	<b>4.1</b>	<b>-0.2</b>
3.2	1.4	3.1	0.0	3.1	1.9	3.8	1.2	<b>4.8</b>	0.8	3.9	0.6	3.4	0.1
3.1	1.9	2.9	0.9	2.6	<b>1.0</b>	3.2	0.8	4.0	0.7	3.6	0.7	3.2	0.1

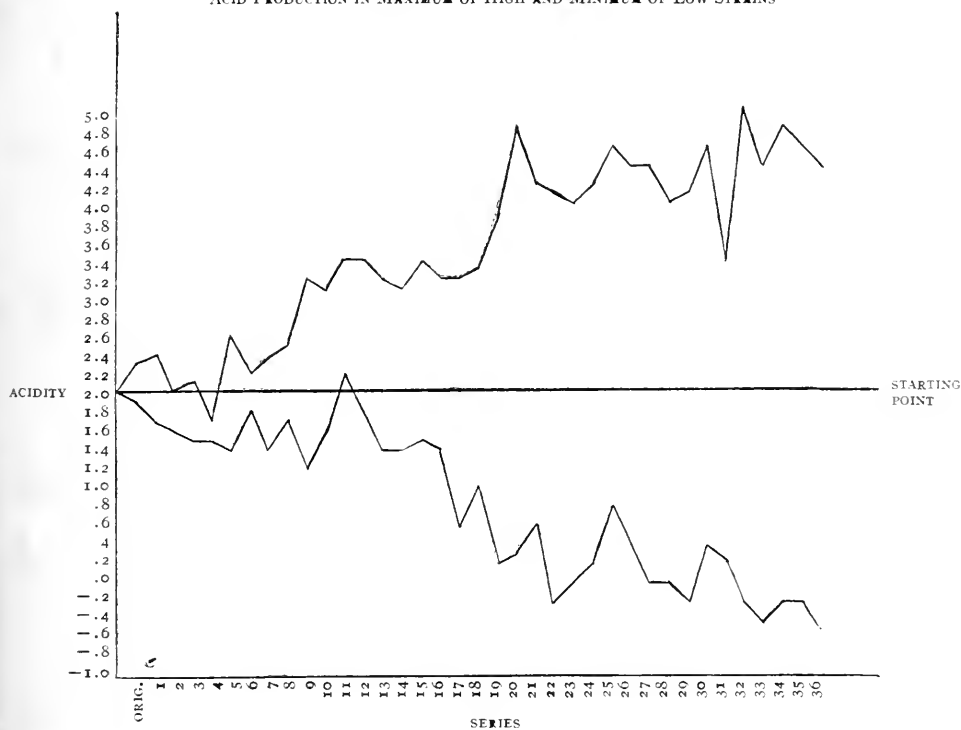
XXIII		XXIV		XXV		XXVI		XXVII		XXVIII		XXIX	
H	L	H	L	H	L	H	L	H	L	H	L	H	L
3.3	<b>0.0</b>	3.5	0.6	4.2	0.8	3.9	0.9	3.8	0.6	3.9	0.5	3.3	0.6
3.8	0.4	2.7	0.9	3.2	<b>0.8</b>	4.3	0.9	2.9	0.7	3.4	<b>0.0</b>	3.6	0.6
3.4	0.1	2.5	0.4	3.0	1.0	3.7	1.2	2.9	0.7	3.8	0.6	4.0	0.2
3.8	0.3	2.6	1.0	<b>4.6</b>	1.6	3.5	0.7	3.7	0.9	3.5	0.3	3.2	-0.1
3.9	0.6	2.8	0.2	4.3	1.0	4.0	0.7	3.6	0.3	3.9	0.3	3.9	0.2
3.2	0.4	<b>4.2</b>	0.3	4.3	1.2	4.0	<b>0.4</b>	4.1	0.7	3.9	0.0	3.9	-0.1
3.9	0.3	2.9	<b>0.2</b>	4.0	1.2	<b>4.4</b>	0.9	3.4	<b>0.7</b>	<b>4.0</b>	0.5	3.8	0.4
3.0	0.6	3.1	0.2	3.5	1.0	4.1	1.1	3.8	1.0	3.6	0.9	3.4	0.1
3.9	0.2	3.1	0.6	3.9	1.3	3.7	1.4	3.6	0.5	3.8	1.1	<b>4.1</b>	0.3
4.0	0.3	3.5	0.3	4.1	1.6	3.9	1.1	3.2	<b>0.0</b>	3.6	1.1	3.7	0.3
<b>4.0</b>	0.3	3.9	0.2	3.7	1.1	3.9	0.8	4.0	0.7	3.0	0.6	3.6	<b>-0.2</b>
3.6	0.4	3.8	0.3	4.1	1.2	3.9	1.2	<b>4.4</b>	0.6	3.1	0.8	3.8	0.0

XXX		XXXI		XXXII		XXXIII		XXXIV		XXXV		XXXVI	
H	L	H	L	H	L	H	L	H	L	H	L	H	L
3.7	1.2	<b>3.4</b>	0.5	4.6	0.2	4.0	0.3	<b>4.8</b>	0.1	3.9	0.5	4.0	0.2
4.1	1.0	2.6	0.3	4.4	0.0	<b>4.4</b>	-0.1	4.0	0.7	3.9	0.1	4.1	0.3
<b>4.6</b>	0.6	2.2	1.1	4.6	0.5	4.1	0.2	4.2	0.4	3.8	0.1	3.5	-0.2
4.4	0.7	2.8	1.2	4.3	0.6	4.0	0.4	3.7	0.0	<b>4.6</b>	0.2	3.6	0.0
4.0	0.7	2.9	0.7	<b>5.0</b>	0.1	4.2	-0.2	4.4	0.0	3.9	0.4	3.2	0.1
4.0	<b>0.4</b>	2.8	1.0	4.0	-0.1	3.7	-0.1	4.4	<b>-0.2</b>	4.2	0.0	3.9	0.6
3.8	1.3	2.5	0.5	4.1	0.3	3.3	0.1	4.5	-0.2	4.4	-0.1	3.6	-0.1
3.1	0.8	3.1	0.9	4.4	0.0	4.1	0.2	4.4	0.3	4.0	0.1	<b>4.4</b>	-0.3
4.1	0.5	3.0	0.9	4.7	<b>-0.2</b>	4.4	0.0	4.2	0.1	3.7	0.3	3.9	0.0
4.0	1.1	2.8	1.2	4.6	0.0	3.5	<b>-0.4</b>	4.1	0.0	4.1	<b>-0.2</b>	4.1	-0.1
3.9	1.5	3.2	0.8	4.0	0.1	3.8	-0.1	3.9	0.1	4.0	0.0	3.8	<b>-0.5</b>
4.3	0.6	3.0	<b>0.2</b>	4.1	0.5	3.7	-0.1	4.5	-0.1	3.8	0.3	3.9	-0.1

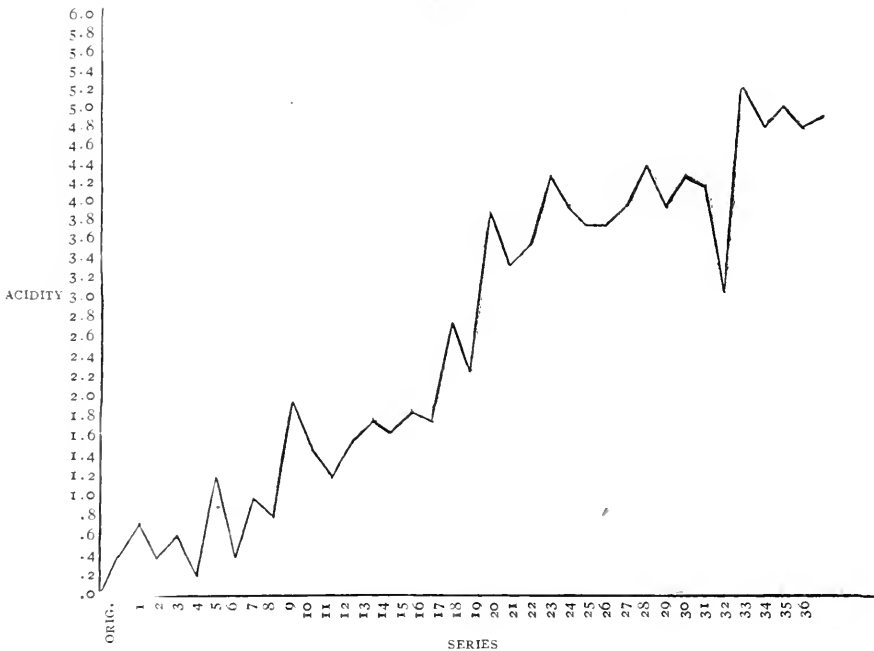
each series are plotted in Chart 3. Although discouragingly close for the first few weeks the curves then begin to diverge very markedly, and by the 20th series the difference between the two is most striking. The extent of the variation effected in the two strains is more easily appreciated by a glance at Chart 4 where the difference in acid-forming power is plotted. The steady and comparatively rapid development of this variation is highly significant, showing how unstable this property is, and how misleading it might be to utilize it as the criterion by which to determine whether a given organism is merely a harmless saprophyte or belongs to one of the most dangerous of bacterial species. As marked as the difference between any diphtheria and pseudo-diphtheria strains in this particular is that between the High and the Low strains at the conclusion of the experiment, for while the former is a rapid and vigorously fermenting organism, producing an extremely

CHART 3.  
ACID PRODUCTION IN MAXIMUM OF HIGH AND MINIMUM OF LOW STRAINS



large amount of acid in dextrose broth, the latter not merely forms no acid but the medium actually becomes alkaline (as a matter of fact this was not observed, but that is inconsequential for the reaction of the medium was made acid to begin with and the acidity was correspondingly diminished in this case). Would one have hesitated to declare the High strain *B. diphtheriae* and the Low strain *B. pseudodiphthericus*, assuming both to be avirulent to guinea-pigs, and their history unknown?

CHART 4.  
VARIATION IN ACID PRODUCTION BETWEEN MAXIMUM OF HIGH AND MINIMUM OF LOW STRAINS  
IN EACH SERIES



In this connection it was of interest to learn what other changes, if any, accompanied this variation in the dextrose fermenting power. The morphology of the two strains was compared with the original parent strain after four transfers on Löffler's inspissated serum. By Neisser's stain, the parent was a slender granular form as it had been originally, and the two daughter strains were likewise granular though rather shorter than the mother, particularly the High strain,

but the difference was by no means marked. The High strain grew appreciably, though not pronouncedly, more vigorously than the Low on Löffler's serum during the first 24 hours and its surface seemed to be somewhat more moist. On agar no differences could be detected between the strains, both growing better than the parent. Their behavior toward the other test carbohydrates is shown in the subjoined table.

TABLE 5.

Strain	Dextrose	Dextrin	Maltose	Saccharose
Parent.....	2.0	1.3	2.2	0.1
High.....	4.4	1.1	1.2	0.6
Low.....	-0.5	1.1	0.3	0.2

It is difficult to understand these changes in the zymogenic property for other carbohydrates, but they nevertheless emphasize the point already made in regard to the instability of this character.

Regarding pathogenicity, the results, unfortunately, were not very satisfying. Twenty-four-hour broth cultures of the Parent, High, and Low strains were inoculated subcutaneously into test-weight guinea-pigs in doses of 0.5 c.c. each, while 200 units (1 c.c.) of antitoxin was simultaneously given intra-peritoneally. The animal receiving the High strain died in two and one-half days with post-mortem lesions typical of diphtheria. The other two animals were quite unaffected except for a little local infiltration at the site of inoculation. It should be mentioned that the Parent strain had originally been extremely virulent for guinea-pigs. As yet I have not repeated the experiment with small quantities of antitoxin and with none at all. Even so, however, the variability of the character is demonstrated.

We may summarize as follows: Separation of the members of the diphtheria group into two or more species on the basis of morphology, staining properties, character of growth, or pathogenicity is not justifiable because of inconstancy. The same applies to immunity reactions, which have the additional objection that their significance as species reactions is as yet unknown. The only character which experience has not shown to be untrustworthy is that of difference in behavior toward carbohydrates. It is unnecessary to discuss here the propriety of establishing species on the basis of but a single differential character, but the foregoing experiments demonstrate

that even these fermentation properties are not species characters since (a) almost every intermediate grade of development can be found by proper methods in different members of the group as they exist in nature, and (b) the extent to which the zymogenic power is exhibited can be readily and markedly altered at will by artificial selection. On the strength of these facts, then, I venture to suggest that the division of the diphtheria group of bacilli into several distinct species is probably based upon a misconception, and that all the forms which have been described under so many different names are but variants of a single species, *B. diphtheriae*, which constitutes the entire group.

It is a pleasure to express my gratitude to Professor E. O. Jordan for many suggestions.

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# THE RESISTANCE OF EMBRYONIC EPITHELIUM, TRANSPLANTABLE MOUSE CANCER, AND CERTAIN ORGANISMS TO FREEZING WITH LIQUID AIR.\*

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THERE appeared recently in the *Lancet*<sup>1</sup> an article by J. E. Salvin-Moore and C. E. Walker on the relationship of cancer cells to the development of cancer, and also a note by J. E. Salvin-Moore and J. O. Wakelin Barratt on the effect of liquid air upon the graftable cancer of mice. Both of these articles deal with experiments made by Moore, Walker, and Barratt in the action of liquid air upon transplantable cancer in mice. The facts and conclusions of each article are practically the same, the first dealing with a transplantable mouse cancer received from Ehrlich, the latter with the Jensen mouse cancer. These authors find that transplantable mouse cancer can be exposed to the freezing of liquid air at  $-195^{\circ}\text{C}$ . for from 20 minutes to half an hour and that such frozen material inoculated into mice is capable in a certain number of instances of producing growing tumors. It is obvious from these articles that the tumors which develop from such grafts are of essentially the same histological appearance as the tumors from which they were taken. Although these authors give prominence to the idea that the freezing in all probability destroys the cancer cells, but leaves intact some virus that stimulates the cells of the host to proliferation with the formation of a new tumor, they consider the possibility of the cancer cells being able to withstand this low temperature. Inasmuch as some bacteria and trypanosomes are said to survive this temperature for a period of 20 minutes, and as normal tissue cells are not supposed to be capable of resisting such temperature, they are inclined to believe that these experiments indicate the presence of a parasite in the cancer tissue.

For the purpose of repeating Moore, Walker, and Barratt's experiments, and for the purpose of control, the following experiments were

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<sup>1</sup> *Lancet*, January 1908, 174, p. 226.

undertaken: A rapidly growing carcinoma of the mouse, which was giving a high percentage of inoculations, known as G 1,532 D was divided into three portions. The first portion was frozen 40 minutes, then injected into 9 susceptible white mice known as Lot 1,561. The second portion was frozen for one hour and 20 minutes, and injected into 14 white mice known as Lot 1,562; and the third portion, without freezing, as control, was injected into five susceptible mice, known as Lot 1,563. The following table shows plainly the procedure followed.

TABLE 1.  
LOT 1,532 D. MARCH 27, 1908.

Lot 1,561, 9 Mice, Tumor Frozen 40 Min.	Lot 1,562, 14 Mice, Tumor Frozen 1 Hr. 20 Min.	Lot 1,563, 5 Mice, Control	Killed
A. nil. B. nil. C. nil. D. nil. E. nil. F. beg. tumor G. large tumor H. nil.	A. nil. B. nil. C. nil. D. nil. E. nil. F. nil. G. died April 28, 1908 H. died May 20, 1908 K. still living 4 nil.	A. beg. tumor B. " " C. " "  } Large tumors E F	March 31, 1908 April 1, 1908 April 3, 1908 April 6, 1908 April 9, 1908 April 14, 1908 June 15, 1908  April 20, 1908 } large April 20, 1908 } tumors

All of the controls developed tumors. A, B, and C were killed on the fourth, fifth, and eighth days respectively. E and F were permitted to grow and on the twentieth day had reached the size shown in the accompanying diagram, when the animals were killed. Among the tumors frozen 1,561 G developed a tumor which was allowed to grow until of large size (see diagram). It was killed on



the thirteenth day. No. 1,562 G developed a more slowly growing tumor, the animal being killed on the thirtieth day (see diagram). No. 1,562 H died on the fifty-second day with a tumor of considerable size (see diagram).

Of the tumors removed in the earlier stages of the process, 1,561 F showed growing tumor cells. In 1,561, of nine mice inoculated with tumor frozen 40 minutes, two tumors developed. In Lot 1,562, 14 mice, material frozen 1 hour and 20 minutes, three developed tumors which were permitted to grow until of considerable size. The controls, five mice, Lot 1,563, gave 100 per cent of rapidly growing tumors.

For the purpose of determining the resistance of trypanosomes to freezing with liquid air, we employed the spleen of rats infected with *Trypanosoma gambiense*.<sup>1</sup> This organism has a degree of virulence which kills normal animals in three days. The infected spleens were taken on the last day of the disease and divided into four portions, one of which was frozen for 20 minutes and injected into two rats, the second portion was frozen 40 minutes and injected into two rats, the third portion was frozen 1 hour and 20 minutes and injected into two rats, and the fourth portion injected direct into two rats as control. The following table shows results.

TABLE 2.  
TRYPANOSOMA MATERIAL FROZEN. RAT A-I. MARCH 31, 1908.

Examination of Blood	Lot 343, 2 Rats, Mat. Frozen 20 Min.	Lot 344, 2 Rats, Mat. Frozen 40 Min.	Lot 345, 2 Rats, Mat. Frozen 1 Hr. 20 Min.	Lot 346, 2 Rats. Control
April 3, 1908.....	1-2 Tryps.	o	o	Died April 3, 1908
April 6, 1908.....	Died	o	o	
April 9, 1908.....		Still living	Still living	

From this it will be seen that *Trypanosoma gambiense* can withstand freezing with liquid air for a period of 20 minutes, although its virulence is somewhat injured, the animals inoculated dying on the sixth and ninth days respectively after inoculation, whereas both the controls died on the third day. Animals inoculated with the organism after freezing 40 and 80 minutes respectively remained uninfected, the organism evidently being killed by exposure to freezing for these periods.

For the purpose of determining what is the resisting power of growing epithelium to freezing, we employed the tissues of young mice embryos, removed aseptically, and after freezing 20, 40, and

<sup>1</sup> The infected rat from which these animals were inoculated was kindly given us by Dr. J. L. Todd.

80 minutes respectively, the materials were injected into mice with suitable direct controls, as shown in the appended Table 3.

TABLE 3.  
EMBRYONIC MOUSE MATERIAL FROZEN IN CONTROL FOR TUMOR 1,532 D. MARCH 27, 1908.

Lot 1,564, 9 Mice, Frozen 20 Min.	Lot 1,565, 8 Mice, Frozen 40 Min.	Lot 1,566, 14 Mice, Frozen 1 Hr. 20 Min.	Lot 1,567, 5 Mice, Control	Killed
A	A	A	A	March 30, 1908
B	B	B	B	April 1, 1908
C	C	C	C	April 3, 1908
D	D	D		April 6, 1908
E	E	E		April 9, 1908
F	F	F		April 14, 1908
1 left	1 left	2 left	all dead	June 3, 1908

For purposes of determining the evidences of growth animals from each group were killed on the fourth, fifth, seventh, tenth, and

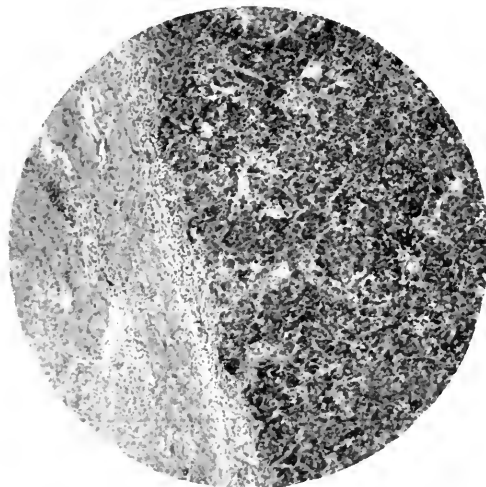


FIG. 1.—Section of mouse tumor, 1,562 G, killed 30 days after inoculation. Shows margin of rapidly-growing, soft, solid carcinoma of the breast, presenting the histological characteristics of the tumor from which the material for inoculation was taken. Material frozen 80 minutes with liquid air.

twelfth days, the implanted embryonic tissue hardened and embedded and subjected to microscopic examination. In each case tumor material, exposed to the same length of freezing and removed on the same day, was at our disposal for comparison. In the case of embryonic tissue it is well known that epithelium transplanted into the subcutaneous tissue of animals of the same species shows distinct evidence of pro-

liferation and growth frequently for a considerable period of time. Such evidences of proliferation can be found in the cells of the implanted embryonic tissue as early as 24 hours after implantation and frequently continue for weeks and even months, producing tumor-like growths which ultimately retrograde and are finally absorbed and removed. To a less extent a similar phenomenon has been observed in transplanted adult epidermis. The epidermis

of guinea-pigs implanted subcutaneously in the guinea-pig, where it is able to form cystlike structures, persists for a considerable period, but is ultimately absorbed. In all the controls of our experiments with embryonic tissue, the embryonic tissue showed evidences of growth. In three of them which were removed on respectively the fourth, fifth, and seventh days, evidences of proliferation of epithelium were distinct. Fig. 2, microphotograph from control removed on the fifth day, shows the characteristic formations of epithelial



FIG. 2.—Growing embryonic epithelium in the subcutaneous tissue of mouse. Characteristic cystlike formations in the epidermis. Proliferating epithelium from the stratum granulosum.

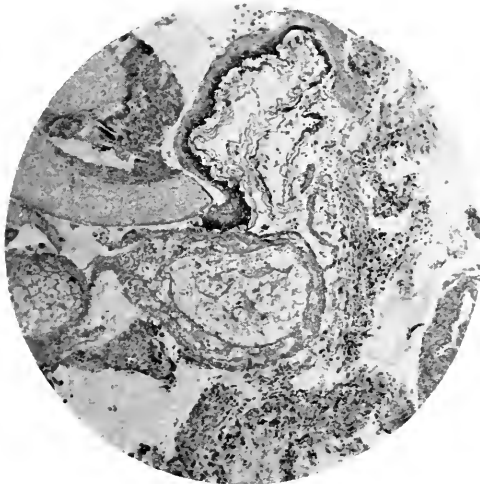


FIG. 3.—Embryonic tissue after freezing 20 minutes. At the margin connective tissue of the host. Remnants of intestinal epithelium, cutaneous epithelium cartilage and muscle, all in an advanced stage of necrosis.

cysts and proliferation of the epithelium into the surrounding connective tissue. All of the embryonic tissue exposed to freezing with liquid air failed to grow and the implanted fragments removed at various periods show complete necrosis of the embryonic tissue and in the later specimens growth of connective tissue and evidences of phagocytosis and advancing absorption. Fig. 3 from a specimen removed on the fifth day after freezing 20 minutes

with liquid air well illustrates the total necrosis of the tissue.

From these experiments it will be seen that embryonic tissue is incapable of withstanding the low temperature developed by freezing with liquid air; that established transplantable cancer of the mouse is capable of withstanding a period of freezing as long as 80 minutes; that *Trypanosoma gambiense* can withstand freezing for 20 minutes, but not for 40 minutes.

#### CONCLUSIONS

1. The cells of transplantable mouse cancer can withstand freezing for a period of 80 minutes and still produce tumors. The percentage of inoculations is greatly diminished, the tumors appear later and grow more slowly than when transplanted directly. They present the same histological picture as the tumors from which they were taken and the controls.

2. Embryonic tissue is killed by freezing with liquid air.

3. *Trypanosoma gambiense* can resist freezing with liquid air for a period of 20 minutes. It is killed at 40 minutes.



## STUDIES ON THE CHEMISTRY OF ANAPHYLAXIS.\*

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UP to the present time much of the work done upon the hypersensitization or anaphylaxis reaction has, of necessity, been of a pioneer nature, and directed with the object of establishing the main facts of the subject in a satisfactory manner, in order that these may serve as a foundation for more speculative investigations into the principles underlying the phenomena observed. The reaction having first been discovered in connection with the experiments on and use of antitoxic serum, and the most obviously important feature of the reaction being its bearing on the clinical use of sera, most of the studies so far recorded have concerned investigations with horse serum, although enough other substances have been tried to indicate that the property of sensitization and intoxication is common to many if not to all proteins. As yet, however, but little has been done in the direction of investigation of the exact chemistry of the substances involved in the reaction and of the chemistry of the reaction itself. The complex nature of the solutions commonly used in anaphylaxis experiments, and the wonderfully minute quantities of substance necessary to produce the phenomena, have both discouraged investigation upon the chemical side of the process. In the following article will be recorded statements that have been made by other investigators which touch upon the chemistry of anaphylaxis, and also certain of the results so far obtained in a series of experiments that have been planned and are being carried on in an attempt to systematically investigate this subject.

The pioneer experiments of Richet (1902), to whom we owe the name anaphylaxis, indicated that the reaction concerned proteins. He observed the phenomenon of hypersensitization in connection with his studies of extracts of *actinia*, and found that only the protein-containing part of the extract exhibited this phenomenon.

\* Received for publication September 8, 1908.

More recent studies by the same investigator<sup>1</sup> demonstrated the impossibility of producing anything resembling anaphylaxis with non-protein substances, such as cocaine and apomorphine. Rosenau and Anderson, in their first paper<sup>2</sup> recorded that the toxic principle of horse serum withstands heating at 60° for six hours, but that it is destroyed by heating to 100° for 15 minutes; that it will pass through a Pasteur-Chamberland filter, that drying does not destroy it, and that it remains in the globulin precipitate that contains the antitoxin as prepared by Gibson's method, and that X-ray exposure ("40 amperes, 4 inches from the tube for 40 minutes") had no evident effect. They support the view that the substance which sensitizes and the substance which poisons are one and the same, and in a later communication<sup>3</sup> stated that they had obtained no results requiring any alteration in this "working hypothesis." They found that the filtrate from horse serum "after precipitation with ammonium sulphate" (concentration not stated) still possesses sensitizing powers; formaldehyde does not appear to modify the sensitizing property, and the sensitizing property did not seem to dialyze through a collodion sac placed in the abdominal cavity of a guinea-pig. They also make the following statement: "We added a number of different ferments, alkaloids, and simpler chemical substances to horse serum in order to modify, destroy or neutralize its toxic action. All these attempts proved unavailing. The following ferments were used: Taka-diastase, pancreatin, rennin, myrosin, invertin, emulsin, pepsin in acid solution, pepsin in alkaline solution, ingluvin, malt and papain. The ferments were added to the horse serum, and allowed to stand at 15° C. over night." In view of the strong inhibitive action that unheated serum exerts upon the proteolytic enzymes, and the slow rate of action of enzymes at 15° C., these negative results with proteolytic enzymes do not imply that the active agent is not of protein nature. Freezing at 15° F. did not modify the toxic substance. They also found that "hemoglobin, egg albumin, milk and extract of peas are quite as active as horse serum. Peptone seems to have slight sensitizing and poisonous properties; leucine and tyrosine none at all. The reaction following the second injections of proteid matter in the guinea-pig appears then to be common to

<sup>1</sup> Richet, *Presse médicale*, 1908, No. 24, p. 185.

<sup>2</sup> *Hygienic Laboratory Bulletin*, No. 29, April, 1906.

<sup>3</sup> *Jour. Med. Res.*, 1907, 16, p. 381.

the higher forms of albuminous substances no matter from what source." Extracts from bacterial masses and yeast cells were found to produce the typical sensitizing and intoxicating effects.

These early experiments indicate strongly that the anaphylaxis reaction, in common with the various reactions of immunity, concerns protein substances, and, like the precipitin reaction, is exhibited by all natural proteins. That the anaphylaxis reaction is essentially dissimilar to the precipitin reaction, is, however, shown by the fact that the blood of either sensitized or intoxicated animals does not exhibit the phenomenon of fixation of complement (Gay and Southard),<sup>1</sup> and Lewis<sup>2</sup> states that the refractory stage following intoxication "depends upon a combination of reactions which is without well-known analogy." Whatever the relation of anaphylaxis to the various phenomena of immunity may be, however, there seems to be at least this in common—all concern proteins.

Gay and Adler<sup>3</sup> have made attempts to isolate from horse serum the protein or proteins concerned in the reaction, with the following results: On adding ammonium sulphate solution to horse serum to the extent of one-third saturation the precipitate obtained (euglobulin) is actively sensitizing but is not toxic. The proteins precipitated in subsequent fractions as the ammonium sulphate is added until saturation, are found to have less and less power to sensitize but are progressively more toxic. The last fraction, obtained on complete saturation, is at least as highly toxic as whole serum and distinctly less sensitizing. These results are believed by Gay and Adler to indicate that the sensitizing and toxic substances in serum are quite distinct from one another, and although they have been unable to obtain a protein that was toxic but not sensitizing this failure is ascribed to the very minute dose of serum that is necessary to produce sensitization. The sensitizing euglobulin which they obtained upon one-third saturation of the serum is looked upon as a pure sensitizing substance, wholly analogous to, if not identical with, the "anaphylactin" found in the serum of sensitized pigs by Gay and Southard. They state that: "It differs from whole horse serum and corresponds to the anaphylactin in the following particulars: (1) it is absolutely nontoxic for sensitive animals; (2) repeated large doses not only

<sup>1</sup> *Jour. Med. Res.*, 1908, 18, p. 407.

<sup>2</sup> *Jour. Exper. Med.*, 1908, 10, p. 1.

<sup>3</sup> *Jour. Med. Res.*, 1908, 18, p. 433.

cause no refractory phase but abbreviate the period of incubation; (3) its injection into sensitive animals not only does not intoxicate, but also produces no refractory (antianaphylactic) phase; (4) it may give rise to sensitization of normal animals in a few days (four or five) as does the transfer of blood from a sensitive animal." They also observed that the ether-soluble constituents of serum, as well as the filtrate from the ammonium sulphate saturation, had neither sensitizing nor intoxicating properties.

These experiments are of particular importance as suggesting that the view that the sensitizing and intoxicating substances are the same, as originally maintained and repeatedly affirmed by Rosenau and Anderson, is not correct. They would seem to indicate rather that the sensitizing substance is either euglobulin itself or some closely related or adherent substance, while the toxic material is some more soluble protein, perhaps an albumin. On the other hand the studies of Vaughan and Wheeler<sup>1</sup> support the view that the sensitizing and the intoxicating substances are both parts of the same protein molecule, quite distinct from one another chemically but each possessing the specificity of the parent molecule. Their experiments were as follows: Egg-white is extracted with boiling absolute alcohol containing 2 per cent of NaOH, by which process the egg-white is split into a soluble poisonous fraction and a non-poisonous fraction which is insoluble in alcohol. The poisonous fraction gives all the protein reactions, except the Molisch test for carbohydrates, and although it is soluble in absolute alcohol Vaughan regards it as a protein on account of its giving all the color reactions. It might well be, however, that it is a soluble peptone or polypeptid containing enough of the different amino acids to give all the usual reactions, and the fact that it is evidently produced by hydrolysis of proteins is in support of this view. Be that as it may, this poisonous substance obtained from the proteins kills normal guinea-pigs with symptoms which, as described by Vaughan and Wheeler, are quite identical with those that appear so characteristically when sensitized guinea-pigs are killed by a second injection of the specific protein to which they have been sensitized. So typical are these symptoms that Vaughan develops the hypothesis that the anaphylaxis reaction is

<sup>1</sup> *Jour. Infect. Dis.*, 1907, 4, p. 476.

due in all cases to this very same poisonous element, which in sensitized animals is split out of the injected proteins by a specific enzyme which has been developed in the cells in response to the first injection of protein. The minimum fatal dose of this poisonous substance is small, "ranging from 8 to 10 or 100 mg. given intra-abdominally, according to the degree of purification reached." There is much disparity between the amount necessary to kill and the amount that suffices to produce symptoms. For example, with one preparation the fatal dose was 70 mg., but 5 mg. developed the earlier stages of intoxication in well-defined form. When given by mouth this poison is without ill effects.

The nontoxic fraction, which is insoluble in alcohol, also gives all the protein reactions and seems to contain all the carbohydrates of the original egg-white. This substance, although itself not poisonous, sensitizes to egg-white when given in doses as low as one or five milligrams, but does not sensitize to itself. This substance which produces sensitization is believed to be a protein, because when treated with uranyl acetate the filtrate left does not sensitize. Vaughan and Wheeler estimate that the second dose of egg-white must be large enough to liberate a fatal dose of poison on cleavage in order to produce death when injected into a sensitized guinea-pig; according to their experiments  $\frac{1}{2}$  c.c. of filtered egg-white is not sufficient to cause a fatal effect, a result not in accord with the experience of others. The poisonous substance does not have the power of sensitizing to egg-white and thus is evidently free from the original sensitizing substance or from unbroken egg-white. Other proteins, including those from bacterial cells, have been found to yield these same poisonous and sensitizing substances, except that no sensitizing substance was obtained with casein or with the protein of the pneumococcus. In a later communication<sup>1</sup> it was stated that gelatin does not yield a poisonous substance, presumably because of its paucity in aromatic radicals. As Vaughan has not worked with pure proteins it cannot be said that these experiments conclusively prove that both sensitizing and poisonous elements are contained in one and the same protein molecule, for it is possible that the boiling with alkaline alcohol separates two different proteins, but the former conclusion seems much the more probably correct.

<sup>1</sup> Symposium on Immunity, American Association for the Advancement of Science, Chicago, Dec. 31, 1907.

## ANAPHYLAXIS WITH PURE PROTEINS.

In developing the investigations of the chemistry of anaphylaxis, it seemed desirable, first of all, to discard the heterogenous mixtures that have been commonly used in these studies, and, as the reaction is one concerning proteins, to work with the purest proteins obtainable that are suitable for the purpose. A number of the vegetable proteins can be obtained in a very pure condition, but these, for the most part, are not available for anaphylaxis studies because of their slight solubility in water. Rosenau and Anderson met with this difficulty when they attempted to sensitize with edestin and excelsin. As most suitable for the purpose, crystallized egg albumin was selected. The most successful way to secure a pure organic substance is, as a rule, crystallization and recrystallization. In egg albumin, from egg-white, we have a protein which crystallizes readily, for a protein, and which can be repeatedly recrystallized for purposes of purification; it also has the advantage of being readily obtained in large amounts, and numerous experiments have shown that crude egg-white is extremely active both in sensitizing and in intoxicating. Gay and Southard<sup>1</sup> found that the relative toxicity of egg-white, horse serum, and cow's milk is in a decreasing scale in the order given.

The crystallization was accomplished according to the method recommended by Hopkins<sup>2</sup> which gives good yields without great difficulty, if eggs but two or three days old are used and the process carried out carefully according to Hopkins' directions. The crystallized egg albumin was recrystallized two or more times, according to the purpose for which it was to be used, then dissolved in a minimum quantity of water, dialyzed until practically free from ammonium sulphate, dried in a current of air at room temperature, ground to a powder, and preserved in a desiccator for use. Prepared in this way the egg albumin may be kept indefinitely in an unchanging condition, and it is easily dissolved in water to form solutions of considerable concentration. Hopkins found that crystallized egg albumin after being recrystallized for a second time is a definite substance of uniform composition, and elementary analysis shows that the product obtained at the first recrystallization is composed of a pure substance,

<sup>1</sup> *Jour. Med. Res.*, 1908, 10, p. 5.

<sup>2</sup> *Jour. Physiol.*, 1900, 25, p. 306.

for it may be divided into several fractions by fractional recrystallization, each of which fractions gives the same analytic figures. Such a soluble, uniform, pure protein offers manifest advantages for the study of the anaphylaxis reaction, and it has been found to be, like whole egg-white, very active both in sensitizing and in intoxicating. In all the experiments with this substance it was used in a 5 per cent solution in distilled water, freshly dissolved for each day's use.

The minimum lethal dose was determined in pigs sensitized 19 days previously by intraperitoneal injection into pigs weighing about 300 gm. of 0.0005 gm. (0.01 c.c. of 5 per cent solution) of egg albumin that had been three times recrystallized. It was found that 0.0005 gm. of the same material given intraperitoneally in the second dose was usually fatal, and doses above this were invariably so. With old pigs the results were less constant, animals sometimes recovering from 0.005 gm., although made very sick by much smaller doses.

By the intravascular route even smaller doses were found to be toxic to sensitized pigs, as shown by the following experiment:

Four young pigs, weighing each 300 gm. were sensitized with 0.02 c.c. of 5 per cent solution of crystallized egg albumin. After 28 days they were given intracardiac injections of dilutions in salt solution of 5 per cent egg albumin solution, with the following results:

No. 1.—Received 0.002 c.c. 5 per cent egg albumin solution (0.0001 gm. of albumin) and showed severe typical symptoms in two minutes; after four minutes was apparently dead, then improved somewhat, but died after 20 minutes. (Autopsy showed that death had not been due to accidents of injection.)

No. 2.—Received 0.001 c.c. of solution (0.00005 gm. of egg albumin) and showed slight but typical symptoms after three minutes, but soon improved. After two hours gave 0.002 c.c. of solution (also intracardiac), which caused symptoms in two minutes and death in 30 minutes. (Autopsy excluded accidental death.)

No. 3.—Received 0.001 c.c. of solution, without any effects. One and one-half hours later gave 0.002 c.c., also without effect. Presumably this pig had not been sensitized.

No. 4.—Received 0.005 c.c. intraperitoneally without any observable effects. Three hours later 0.001 c.c. was given by intracardiac injection, causing symptoms in two minutes, and death in six minutes. (Autopsy excluded accidental death.)

The sensitizing dose of this same preparation is also extremely small, as shown by Table 1.

From this series it is seen that one-millionth of a cubic centimeter of 5 per cent solution of pure egg albumin, or *one-twentieth of a millionth of a gram of protein*, will sensitize a guinea-pig enough so

TABLE 1.

	AMOUNT OF SENSITIZING DOSE		SIZE OF SECOND DOSE*	INTERVAL BETWEEN INJECTIONS	RESULT
	5% sol.	Dry wt.			
1.....	0.5 c.c.	0.025 gm.	0.25 gm.	21 days	Death
2.....	0.05	0.0025	0.05	21 "	"
3.....	0.005	0.00025	0.025	21 "	"
4.....	0.0005	0.000025	0.2	21 "	"
5.....	0.00005	0.0000025	0.25	19 "	"
6.....	0.00002	0.000001	0.2	19 "	"
7.....	0.00001	0.0000005	0.2	19 "	Severe symptoms
8.....	0.000005	0.00000025	0.2	19 "	"
9.....	0.000002	0.0000001	0.2	19 "	Slight
10.....	0.000001	0.00000005	0.2	19 "	Moderate

\* When not otherwise stated all injections were made into the peritoneal cavity.

that distinct and typical symptoms are produced after a second injection of the same material, while one-fifty-thousandth of a cubic centimeter of solution containing but *one-millionth of a gram of protein sensitizes fatally*.

Rosenau and Anderson<sup>1</sup> found that one-millionth of a cubic centimeter of horse serum will sometimes, but not always, sensitize, although in their series of experiments upon this point the smallest fatally sensitizing dose recorded was one-thousandth of a cubic centimeter. Gay and Adler<sup>2</sup> found that of their sensitizing proteid of serum (euglobulin) an amount corresponding to 0.01 c.c. of the original serum caused fatal sensitization, but sensitization with 0.0001 c.c. and 0.00001 c.c. caused symptoms without fatal results; however, they do not state just what quantity of euglobulin these figures represent. In any case, however, it would seem that the pure egg albumin is probably more actively sensitizing than the other substances that have been used in anaphylaxis studies.

The extremely minute quantities of crystallized egg albumin that were found effective in sensitizing and intoxicating, made comparison with unpurified egg-white desirable, and the following experiments were performed: Egg-white was diluted with an equal volume of water, beaten, and filtered. To a sample of this filtrate ten volumes of alcohol were added, the precipitated protein dried and weighed, and the solution was found to contain 6.3 per cent of protein. The minimum sensitizing dose of this solution was found to be (for 300-gram guinea-pigs) about 0.0001 c.c. (or 0.0000063 gm.). Doses smaller

<sup>1</sup> *Hygienic Laboratory Bulletin*, No. 29, 1906.

<sup>2</sup> *Jour. Med. Res.*, 1908, 18, p. 440.



than this produced no sensitization whatever, while fatal results were only obtained when 0.001 to 0.0005 c.c. were used for sensitizing.

The minimum intoxicating dose (intraperitoneal) of this solution, for pigs sensitized with unpurified egg-white, was found to be about 0.01 c.c. or 0.00063 gm.; but this amount produced but slight symptoms, fatal effects only resulting from doses as large as 0.04 c.c. or 0.0025 gm. of egg-white proteins.

Contrasted with the crystallized egg albumin, therefore, the unpurified mixed proteins of egg-white are about one-fifth as toxic for sensitive guinea-pigs, and but about one-hundreth as actively sensitizing.

This indicates that the toxicity of this purified protein is perhaps even greater than that of the whole egg-white, as if there might be inhibiting substances present in the untreated egg-white. The fatal dose of this protein, one-half of one milligram, is far smaller than the fatal dose of the poisonous fraction of egg-white prepared by Vaughan and Wheeler, which they give as "from 8 or 10 to 100 mg. given intra-abdominally, according to the degree of purification reached." They estimate that to liberate a fatal dose of their poisonous substance from egg-white, more than 60 mg. of the protein must undergo cleavage in the body, whereas these experiments show that one-half a milligram of pure protein furnishes a fatal dose for 300-gram pigs when given intraperitoneally and one-tenth as much is fatal when injected directly into the blood.

#### THE TOXIC ELEMENT OF PROTEINS.

These experiments may also be taken to indicate that both sensitization and intoxication may be produced by one and the same sort of protein molecule. Indeed, the sensitizing and intoxicating effects have been produced with such extremely minute quantities of protein that it seems not unwarranted to maintain that these experiments settle this point conclusively. As a repeatedly recrystallized egg albumin, giving every evidence of chemical purity, is capable of causing sensitization in doses of one twenty-millionth of a gram, and of killing sensitized pigs in doses of one-twentieth of a milligram, it is evident that if either of these effects were due to something other than the egg albumin itself, this contaminating substance must have

a potency beyond the bounds of the imagination. It is of course possible that in the case of horse serum the sensitization and the intoxication are effected by different proteins, as the work of Gay and Adler suggests—one cannot deny this possibility in the present incomplete state of our knowledge—but in the case of crystallized egg albumin such a possibility may be reasonably excluded.

Granting this, we have, among others, two possibilities—either the whole protein molecule accomplishes both the sensitization and the intoxication, or one part of the molecule does the sensitizing and another part of the complex structure produces the intoxication. Vaughan's work is, of course, in favor of the latter idea, for he seems to have been able to split out of impure proteins substances that will sensitize but not intoxicate, and substances that will intoxicate but not sensitize.

If the intoxication that follows the second injection of protein into animals that have been sensitized by a previous injection depends upon a cleavage of the proteins with liberation of toxic groups, we have several reasons for suspecting that the intoxicating element will be found to bear a close relation to the aromatic radicals of the protein molecule. In the first place we have the fact that compounds containing the benzene ring are commonly poisonous, from the simplest derivatives such as phenol and aniline up to complex substances such as the terpenes. Also it is known that in the digestion of proteins, bactericidal substances are formed, and these are apparently the derivatives of aromatic radicals of the proteins. Again, in their important studies upon the precipitins, which will be considered more extensively subsequently, Obermayer and Pick found that the specificity of the precipitin reaction seems to depend upon the aromatic radicals of the protein, an opinion supported by Fleischmann's observations on the effect of tryptic digestion upon serum. Finally, we have the statement of Vaughan that the toxic substance split off the proteins in his experiments, contains the aromatic radicals, and that gelatin, which is of all proteins the poorest in aromatic radicals, does not yield a toxic portion. In view of these facts it seems probable that gelatin should be defective in toxicity, and perhaps entirely unable to cause sensitization, and such has been found to be

the case.<sup>1</sup> Guinea-pigs were injected with doses of from 5 c.c. down to  $\frac{1}{32}$  c.c. of 4 per cent commercial gelatin solution, and after two weeks or more were given 8 to 10 c.c. of a similar solution intraperitoneally. In not a single case were any symptoms whatever produced by the second dose. Animals sensitized to egg-white or to bovine serum did not show any symptoms when injected with gelatin solution; and conversely, injection with gelatin did not sensitize to egg-white or bovine serum. Injection of gelatin solution into animals sensitized with egg-white did not make the animals at all refractory to injections of egg-white 24 hours later.

Although gelatin possesses of the aromatic compounds commonly found in proteins only a small quantity of phenyl alanine, and no tryptophane or tyrosine (probably), yet addition of tyrosine equal in amount to 6 per cent of the gelatin in a 4 per cent gelatin solution, did not produce a compound at all capable of causing either sensitization or intoxication, when used itself for both injections, or when used either as the first or second injection in connection with an injection of egg albumin or horse serum. This negative result was, of course, to be expected, for even if the toxicity of proteins depends upon their aromatic radicals these radicals probably act only in combination with many others in a large fragment of the entire protein molecule, and not as free radicals as in these experiments.

#### THE EFFECT OF HEAT UPON THE SUBSTANCE THAT IS CONCERNED IN ANAPHYLAXIS.

An objection that may be advanced against these experiments (one that Dr. J. F. Anderson has kindly made in a personal communication) is that gelatin is heated in its preparation, and heat destroys both sensitizing and intoxicating effects of proteins, at least those in serum. Concerning the effects of heat upon horse serum Anderson and Rosenau state in their latest publication<sup>2</sup> that "the sensitizing effect of horse serum is greatly influenced by heat and almost entirely disappears when the serum is heated to 100° C. for one hour." As to the effect of heat upon the intoxicating constituent, heating to 70° C. for one hour does not seem to diminish

<sup>1</sup> A preliminary report upon this topic has been published in the *Jour. Amer. Med. Assoc.*, 1908, 50, p. 527.

<sup>2</sup> *Jour. Med. Res.*, 1908, 19, pp. 38, 42.

appreciably its poisonous properties, but these seem to be somewhat affected at 80° C. for one hour. At 100° C. for one hour the toxicity apparently disappears. Gay and Adler found that the toxicity is decreased if the serum is heated at 90° for 15 minutes, and destroyed at 100° for 15 minutes; sensitization is accomplished by serum heated at 100° for 15 minutes, but not if heated 45 minutes. In order to prevent coagulation Rosenau and Anderson diluted the serum with three parts of distilled water, while Gay and Adler used nine parts of 0.85 per cent salt solution, but both note that at 100° the solution becomes more or less milky or opalescent.

Now while it cannot be denied that heat *may* in some way alter the gelatin molecule so that it cannot produce sensitization, yet there is very good reason to hold that the results obtained by heating horse serum cannot be applied to gelatin solutions, and that it is more probable that the inactivity of gelatin depends upon its chemical composition rather than upon the effect of the heat used in the preparation of gelatin from collagen by hydration. Gelatin is a member of a peculiar class of colloids forming "heat reversible gels;" that is, they are liquefied by heat and return to the gel form when cooled, which process can be repeated many times if no acids or alkalis are present to hydrolyze the gelatin. The application of heat up to 100° to gelatin, therefore, does not permanently change its physical properties, and it is just as capable of forming collodial solutions as it was before. On the other hand, application to serum of temperatures considerably below the boiling point produces a severe physical change in the proteins by which they are coagulated and rendered insoluble; dilution of the serum does not alter this process except to prevent the coagulated particles from aggregating in large flocculi, for it does not require a very high power of the microscope to show that the opalescent heated serum dilution contains the protein in a granular, coagulated form. Now the behavior of such a protein when injected into the abdominal cavity is undoubtedly quite different from that of a protein that has not been coagulated. The uncoagulated protein is in a state of colloidal solution, and in a dissolved condition is absorbed unchanged, in evidence of which is the well-known fact that foreign proteins injected into the abdominal cavity of animals are excreted unchanged in the urine. This process of absorption should be quite the same with unheated serum and with gelatin solutions

which cannot be coagulated by heat. On the other hand, the situation with heat-coagulated proteins is quite different, for the protein does not then dissolve in the peritoneal fluids, but is, presumably, taken up like any other finely divided particles by the leucocytes and the endothelial cells, there to be digested and fundamentally altered before reaching the general circulation and the cells (which-ever they may be) where the protein produces its effect. In other words, the fact that boiling destroys the sensitizing and intoxicating properties of heat-coagulable proteins is no evidence that it will affect in a similar way proteins that are not coagulable by heat, and hence the failure to secure sensitization or intoxication by gelatin is probably due not to the heating it has undergone, but rather is because of the chemical composition of the gelatin itself.

In support of the view that the effect of heat in interfering with the sensitizing and poisonous action of proteins depends entirely upon its effect in rendering the proteins insoluble, rather than by the production of chemical changes in the protein, may be cited the results of experiments with milk. The chief protein of milk, caseinogen, is not coagulated by heating to boiling, except for a small portion which dries out upon the surface to form the "skin" of scalded milk. As can be seen by the following experiments, heating milk to 100° for a considerable length of time does not destroy either its sensitizing or its intoxicating power.

TABLE 2.

	First Injection	Interval	Second Injection	Result
1.....	0.2 c.c. raw milk	21 days	5 c.c. raw milk	Death in 6 hours
2.....	0.2 " " "	21 "	10 c.c. milk (sour) heated 30 min. at 100° C. (partly curdled)	No symptoms. 24 hours later injected 5 c.c. raw milk causing only slight symptoms
3.....	0.2 " " "	21 "	10 c.c. fresh milk heated 30 min. at 100° C.	Death during night, after 6 hours
4.....	0.5 c.c. milk heated to 100° C., 5 min.	21 "	10 c.c. fresh milk heated to 100° C. for 40 min.	Death after 6 hours
5.....	0.5 c.c. milk heated to 100° C. for 25 min.	21 "	10 c.c. raw milk	Death in 15 min.
6.....	1.0 c.c. milk heated 45 min. to 100° C.	21 "	10 c.c. raw milk	Death in 6½ hour
7.....	1.0 c.c. raw milk	19 "	10 c.c. milk heated to 100° C. for 30 min.	Death in 75 min.
8.....	1 c.c. raw milk	19 "	10 c.c. milk heated to 100° for 40 min.	Slight symptom

A particularly interesting corroboration is furnished by experiment No. 2 of this series, in which the milk had become soured before being heated; the usual results of heating soured milk followed,

the caseinogen being converted into casein and precipitating out, and this milk alone lost its toxicity upon heating.<sup>1</sup> These experiments seem to show conclusively that whether a protein will be deprived of its toxicity by heating depends solely upon whether it is or is not coagulated by heat.

An additional bit of evidence pointing in the same direction is the following: Serum albumin, in contrast with egg albumin, can be permitted to stand for some time in strong alcohol by which it has been precipitated, without losing its solubility in water. If serum albumin is thus precipitated by alcohol and left standing a few hours, thoroughly washed with alcohol, dried and redissolved in water, it is found to be quite as actively toxic to sensitized guinea-pigs as fresh serum. On the other hand, if solutions of egg albumin are treated with alcohol in the same way, the albumin becomes almost entirely insoluble, and if it is washed free from alcohol, ground up to a fine emulsion in water, and injected into sensitized pigs, it produces no or very slight symptoms, and commonly it does not even render the animal refractory to a subsequent injection of egg albumin. This fact indicates, at least, that the mere process of coagulation of proteins does not seriously impair their toxicity, provided they are still soluble in water, and that the effect of coagulation of proteins upon their toxicity depends entirely upon the loss of solubility which the proteins suffer.

In this connection may be stated the results of experiments made to determine the effect of heat upon the sensitizing and intoxicating properties of pure egg albumin. On account of the apparent effect of coagulation upon the intoxicating action of serum and milk, these experiments were performed in pairs, to one of each of the pairs of heated albumin solution a drop of very dilute acetic acid being added after the heating was completed, to increase the degree of aggregation of the coagulated proteins. The results were as appear in Tables 3 and 4.

These experiments indicate that crystallized egg albumin is affected by heat in much the same way as is serum. The sensitizing power is not altogether destroyed at boiling temperature even with

<sup>1</sup> Apparently even with this curdled milk enough was absorbed, coagulation not having been absolutely complete, to render the animal refractory to an injection of raw milk 24 hours later.

TABLE 3.

EFFECT OF HEAT ON SENSITIZING POWER OF CRYSTALLIZED EGG ALBUMIN.

For each injection 5 c.c. of a 1 per cent solution of egg albumin, heated for 15 minutes at the temperature specified, was used. The second dose was given after an interval of 19 days, and consisted of 3 c.c. of unheated 5 per cent egg albumin solution.

Temperature		Symptoms in 10 minutes.		Results	
1.	100°	"	"	Death in 10 minutes.	
1A.	100°+acetic	"	20 "	never severe, and animal recovered.	
2.	90°	"	23 "	Death in 100 minutes.	
2A.	90°+acetic	"	36 "	but only slight and animal recovered.	
3.	80°	"	10 "	Death in 30 minutes.	
3A.	80°+acetic	"	10 "	" " 25 "	
4.	70°	"	10 "	" " 40 "	
4A.	70°+acetic	"	10 "	" " 28 "	

TABLE 4.

EFFECT OF HEAT ON INTOXICATING POWER OF CRYSTALLIZED EGG ALBUMIN.

The pigs had been sensitized 28 days previously with 0.2 c.c. of unheated egg albumin solution. They were injected with 2 c.c. of 5 per cent egg albumin solution diluted with 10 c.c. of water, which had been heated as indicated for 15 minutes.

Temperature		Results	
1.	90°	Slight or doubtful symptoms; recovered.	
1A.	90°+acetic	No symptoms.	
1B.	90°	Slight symptoms after 2 hours; recovered.	
2.	80°	Symptoms in 22 minutes.	Death in 4½ hours.
2A.	80°+acetic	" " 20 "	" " 2 "
3.	70°	" " 20 "	" " 25 minutes.
3A.	70°+acetic	" " 10 "	" " 45 "

serum, provided large enough quantities of protein are injected, for I have obtained fatal sensitization of pigs with 2 c.c. of serum diluted to 10 c.c. with water, after heating to 100° for 45 minutes. This probably means either that minute quantities of protein escape coagulation through partial hydrolysis, or else that through the process of digestion of the coagulated material by the cells in the peritoneal cavity, a small quantity enters the blood in a soluble form still capable of sensitizing.<sup>1</sup> Toxicity, which requires a much larger dose to make it manifest, disappears at a lower temperature. Increasing the size of the coagulated particles, and perhaps also the

<sup>1</sup> It may be mentioned that in these experiments every necessary precaution was taken against introducing traces of unheated protein, which might cause sensitization in even minute quantities.

completeness of the coagulation by use of acetic acid, seems to augment slightly, but not very consistently, the effect of heat.

#### ANAPHYLAXIS WITH VEGETABLE PROTEINS.

In connection with the consideration of the influence of aromatic radicals of proteins upon their toxicity to sensitized animals, the alcohol-soluble vegetable proteins, zein and gliadin, were examined. The protein gliadin from wheat is, as compared with most proteins, poor in aromatic amino-acids, although it has much more than gelatin, while zein is particularly rich in tyrosine as is shown by the following table of results of hydrolysis of these proteins.

TABLE 5.

	Gliadin* per cent	Zein* per cent	Gelatin† per cent	Egg Albumin‡ per cent
Glycocol.....	0.13	0	16.5	0
Alanine.....	1.33	2.23	0.8	2.1
Valine.....	?	0.20	present (?)	?
Leucine.....	6.30	18.60	2.1	6.1
Proline.....	9.82	6.53	5.2	2.25
Phenylalanine.....	2.70	4.87	0.4	4.4
Aspartic acid.....	0.25	1.41	0.56	1.5
Glutamic acid.....	33.81	18.28	0.88	8.0
Serine.....	0.06	0.57	....	....
Tyrosine.....	1.19	3.55	0	1.1
Arginine.....	2.22	1.16	0.3	1.5
Lysine.....	0	0	5-6	....
Histidine.....	0.39	0.43	0.4	....
Tryptophane.....	present	0	0	present

\* Osborne and Clapp, *Amer. Jour. Physiol.*, 1908, 20, p. 494.

† Fischer, Levene, and Aders, *Ztschr. physiol. Chem.*, 1902, 35, p. 70.

‡ Abderhalden and Pregl, *ibid.*, 1905, 46, p. 24.

The preparations of zein and gliadin were kindly furnished me by Dr. Thomas B. Osborne, and were thoroughly purified by reprecipitation. As they are not soluble in water, they were dissolved in 0.2 per cent NaOH solution to a concentration of 4 per cent of protein; this was then diluted as much as the exigencies of the experiment in hand warranted, commonly one-half, and  $\text{NaHCO}_3$  added until signs of precipitation began, for the purpose of converting as much as possible of the NaOH into the less irritating  $\text{Na}_2\text{CO}_3$ . The results of the experiments are given in Tables 6 and 7.

These experiments would seem to indicate that gliadin, with its low content of tyrosine and phenyl alanine, is weak in either intoxicating or sensitizing properties, presumably the former; however,



TABLE 6.  
ZEIN ANAPHYLAXIS.

	First Injection	Interval	Second Injection	Results
1.....	0.04 gm.	18 days	0.12 gm.	Moderate symptoms; recovered
2.....	0.02	18 "	0.10	Severe symptoms; recovered
3.....	0.01	18 "	0.12	Severe symptoms; recovered
4.....	0.005	18 "	0.10	Slight symptoms; recovered
5.....	0.02	17 "	0.20	Death, 1 hour
6.....	0.004	17 "	0.25	Death, 40 minutes
7.....	0.002	18 "	0.20	Death, 12 hours
8.....	0.0004	17 "	0.20	Severe symptoms; recovered
9.....	0.0002	18 "	0.20	Severe symptoms; recovered
10.....	0.01	25 "	0.20	Death in 6 hours
11.....	0.01	25 "	0.20	Death in 6 (?) hours
12.....	0.005	25 "	0.15	Severe symptoms; recovered

NOTES.—The first four animals received for the second dose a solution of zein in 0.2 per cent of NaOH with a little chloroform, that had been standing in the ice chest for 18 days, which probably accounts for the less severe symptoms in this series. To make sure that the symptoms and death were not due to the alkalinity of the solution, control pigs received the same dose of each preparation used in these experiments, but only transient symptoms of peritoneal irritation were ever produced. Animals 8, 9, and 12 were each given a third injection of zein 24 hours after the intoxicating dose, but in none were symptoms observed, showing that zein produces a refractory stage in animals recovering from intoxication. In all experiments the onset of symptoms was rather slow, as compared with serum sensitization, which is presumably due to the relatively poor solubility of zein in the body fluids.

TABLE 7.  
GLIADIN ANAPHYLAXIS.

	First Injection	Interval	Second Injection	Results
1.....	0.04 gm.	18 days	0.20 gm.	Severe symptoms; recovered
2.....	0.01	19 "	0.20	" " "
3.....	0.005	17 "	0.10	Slight " "
4.....	0.001	18 "	0.12	" " "
5.....	0.02	19 "	0.20	Moderate " "
6.....	0.02	19 "	0.20	" " "
7.....	0.02	20 "	0.20	" " "
8.....	0.01	14 "	0.20	" " "
9.....	0.004	14 "	0.20	No symptoms
10.....	0.01	14 "	0.16	No symptoms
11.....	0.2	23 "	0.20	Died, 45 minutes
12.....	0.2	23 "	0.20	Moderate symptoms; recovered
13.....	0.04	14 "	0.20	No symptoms
14.....	0.02	14 "	0.20	No symptoms
15.....	0.2	20 "	0.25	No symptoms
16.....	0.2	20 "	0.25	No symptoms
17.....	0.004	26 "	0.25	Slight symptoms; recovered
18.....	0.02	26 "	0.25	" " "
19.....	0.08	26 "	0.25	" " "
20.....	0.16	26 "	0.25	" " "

the preparation of gliadin used was so readily thrown out of solution that it is possible that part of this apparent inertness is due to insolubility, although I do not consider this probable. With zein there was no difficulty in securing effective sensitization and fatal intoxication with all the characteristic symptoms, and the zein is little more soluble than the gliadin. This action of zein is particularly inter-

esting in view of the absence of tryptophane from the zein molecule, and would seem to indicate that this aromatic radical, at least, is not essential for intoxication by proteins.

Other experiments showed that zein does not sensitize to gliadin, and conversely. This was to be expected in view of the fact that with the precipitin reactions the vegetable proteins show a species specificity fully as sharp as is observed among animal proteins, and according to Gasis<sup>1</sup> even more distinct.

Although all the experiments described above, together with some facts to be brought out later, seem to point to the aromatic radicals of the protein molecule as being in some way essentially concerned in the intoxicating effects which proteins exert upon sensitized guinea-pigs, yet it is rather difficult to believe that it is actually the aromatic radicals, or for that matter any other part of the protein molecules that are injected into the guinea-pig for the second dose, that act as the fatal poison. If we consider the quantity of pure protein that is sufficient to kill a sensitized guinea-pig, but 0.00005 to 0.0001 gm., and try to imagine what could be derived from that amount of protein that would produce fatal intoxication, we certainly find it a difficult problem. If there is a poisonous group which is liberated from the second dose of protein, whether by a special enzyme as Vaughan suggests or by some other process, it must be something most wonderfully poisonous, and it is very difficult to understand from what we know of the structure of the protein molecule just how it could produce so exquisite a poison and what the structure of this remarkable poison might be. Of course we may assume that under the special conditions of sensitization the cells become peculiarly permeable, or have an especial affinity for the unknown toxic substances, so that we cannot compare them with the ordinary poisons which act in animals not specially sensitized to them. Or we may perhaps legitimately imagine that the second dose injected into sensitized guinea-pigs causes their cells to produce from their own constituents or from the constituents of the blood the poisons that produce the fatal results. As an analogy for this latter hypothesis we have the fact that when a serum containing specific precipitins is added to a small quantity of the homologous protein the precipi-

<sup>1</sup> *Berl. klin. Wchnschr.*, 1908, 45, p. 358.

tate that forms is derived chiefly from components of the specific serum and not from the protein with which it reacts.

However, the rapidity with which the intoxication occurs once the second dose of serum has entered the blood, oftentimes but a fraction of a minute after intracardiac injections, speaks somewhat against the idea that any complicated process has to go on before the toxic substance is prepared, and consequently is in favor of the view that it really is the protein itself or some substance that can be readily formed from it that exerts the lethal effect. Much more evidence must be secured before we can determine this point.

#### EFFECT OF IODIZATION OF PROTEINS.

At the present time the determined facts concerning the anaphylaxis reaction indicate that it may be something quite different from the known reactions of immunity, but in its limitation to the protein substances it has much in common with them, and in the fact that specific reactions can be produced with a great variety of proteins it recalls in particular the precipitin reaction. In connection with the problems of the precipitin reaction, Obermayer and Pick<sup>1</sup> have made interesting and important observations, which may be briefly summarized as follows: The proteins seem to contain two fundamental groupings of amino-acid radicals, one of which determines the specificity of the precipitin reaction for the species of animal from which the protein comes; and another *konstitutive Gruppierung* which determines the specificity that is characteristic for the protein itself and which varies with the total structure of the entire protein molecule. The former of these, the species specificity, is much more firmly established, and resists alterations in the molecule much longer than the latter. To illustrate, if an animal is immunized to normal bovine serum its serum will not precipitate bovine serum that has been altered by heat. However, if immunization is done with heated bovine serum, the animal's serum will react not only with heated bovine serum, but also with normal bovine serum and with a whole series of its splitting products with which the serum of animals immunized to normal bovine serum will not react. But the serum against heated bovine serum is just as specific as the other,

<sup>1</sup> *Wien. klin. Wchnschr.*, 1906, 14, p. 327.

in respect to species, for it will react only with proteins of bovine origin. Similar effects may be produced by altering proteins in various ways, such as converting them into alkali- or acid-albuminates, or acting upon them with formaldehyde, toluol for a long period of time, or by partial oxidation with alkaline permanganate solution. In other words, it is very easy to alter a protein so that it produces precipitins for proteins other than itself, provided these proteins come from the same animal species as it comes from itself. On the other hand, the specificity for species seems to be affected only when the aromatic radicals of the protein molecule are acted upon in various ways. This is most readily done by introducing such radicals as iodine,  $\text{NO}_2$ , and  $\text{N}=\text{N}$ , into the protein molecule, where they are known to enter specifically the benzene rings of the aromatic radical. It was found that such altered proteins produce precipitins that are specific for the form of protein used in immunizing, but not at all specific as regards the species from which the protein is derived; e. g., iodized bovine serum produces in immunized rabbits precipitins that will react only with iodized proteins, but they react with iodized proteins of any source, whether from egg-white, horse serum, or serum of some other animal, even of iodized rabbit proteins. On the other hand, such modified proteins are quite specific toward themselves, that is, precipitins for iodized proteins will not react with diazotized proteins, and conversely. Finally, if radicals are introduced into amino-acids other than those entered by iodine, etc. the species specificity is not lost; e. g., diazo-benzol radicals enter the protein molecule, probably into the histidine group, and the resulting diazobenzol-protein produces precipitins specific for itself and which also exhibit specificity for the particular animal species from which the protein was derived.

These important observations have not yet, so far as I can learn, been subjected to either re-examination or to extension, and it seemed highly desirable to learn to what extent they might apply in the anaphylaxis reaction. For this purpose the following experiments were performed:

*Iodized serum, Preparation I.*—Preparation I of iodized bovine serum was prepared according to the procedure recommended by Blum,<sup>1</sup> as follows: To 50 c.c.

<sup>1</sup> *Ztschr. physiol. Chem.*, 1899, 28, p. 288.

bovine serum was added 1.5 gm. KI, 0.75 gm. iodine, and 0.05 gm. potassium iodate, and the mixture was made faintly alkaline with 2 c.c. of 10 per cent sodium bicarbonate solution. Kept in water bath at 45° for two hours, then at 38° for 18 hours; then added one-fifth as much of the I-KI solution as first used, and heated at 45° for one hour. There was a small amount of precipitate, which was removed by filtration, and the clear filtrate was dialyzed through collodion against cold running water for 48 hours. Analysis of the iodized serum solution left in the dialyzer showed it to contain 4.24 per cent by weight of protein coagulable by alcohol (dilution through endosmosis had occurred), and this protein contained 8.89 per cent of iodine. As completely iodized serum albumin contains 12 per cent of iodine by weight (Kurajeff),<sup>1</sup> and serum globulin contains 13 to 14 per cent (Hopkins) it is evident that this preparation represents an incompletely saturated iodo-protein. It also differs from the typical iodo-proteins in being soluble in water, and in giving Millon's reaction, but resembles them in that the iodine is so firmly bound that it cannot be detected by the usual reactions.

With this preparation the following results were obtained:

TABLE 8.

	First Injection	Interval	Second Injection	Results
1.....	5 c.c. iodized serum	16 days	5 c.c. iodized serum	Death in 45 min.
2.....	0.01 " "	16 "	5 " "	Severe symptoms
3.....	0.02 " "	24 "	7 " "	Death in 90 min.
4.....	0.02 " "	30 "	7 " "	Death in 20 min.
5.....	0.1 " "	16 "	5 bovine serum	No definite symptoms
6.....	0.001 " "	16 "	5 " "	Death in 35 min.
7.....	0.02 " "	30 "	6 " "	Death in 30 min.
8.....	0.02 " "	24 "	5 " "	Severe symptoms
9.....	0.02 " "	21 "	1 egg-white	No symptoms
10.....	0.02 " "	60 "	0.25 gm. egg albumin	No symptoms
11.....	0.02 " "	21 "	0.2 gm. zein	No symptoms
12.....	0.02 " "	21 "	3 c.c. dog serum	Slight symptoms
13.....	0.02 " "	18 "	3 " "	No symptoms
14.....	0.02 " "	19 "	5 " "	No symptoms
15.....	0.02 " "	18 "	3 horse serum	No symptoms
16.....	0.05 bovine serum	21 "	5 iodized serum	No symptoms
17.....	0.05 " "	22 "	5 " "	No symptoms
18.....	0.05 " "	23 "	5 " "	No symptoms
19.....	0.05 " "	20 "	5 " "	No symptoms
20.....	0.01 " "	21 "	5 " "	Death in 50 min.

These results indicate that this particular incompletely iodized serum possesses the property of fatally sensitizing guinea-pigs to normal bovine serum and to itself, but it does not sensitize to foreign proteins. What is of particular interest is that it does not intoxicate pigs sensitized with normal bovine serum (only one exception observed), although quickly killing pigs sensitized to itself. The pigs that failed to react to the iodized serum (Nos. 16-19) were given 5 c.c. each of normal bovine serum 24 hours later and all reacted distinctly (two moderately, two severely) but recovered, indicating that the iodized serum had not conferred the refractory phase commonly

<sup>1</sup> Mann, *Chemistry of the Proteins*, p. 231.

seen in animals recovering from an intoxicating dose, although the animals were perhaps somewhat protected by the iodized serum since none of them died. The animals sensitized with iodized serum that recovered from injections of heterologous proteins (Nos. 9 to 15), were given 24 hours later normal bovine serum, and all developed more or less severe symptoms, only one (No. 14), however, dying.

*Iodized serum, Preparation II.*—200 c.c. bovine serum, which contained considerable hemoglobin, was treated as in the previous preparation, except that the temperature at one time rose to 50°. There was a large amount of precipitate, which was, unfortunately, not saved, only the filtrate being dialyzed. The dialyzed filtrate represented a dilute protein solution, the ammonia from 5 c.c. decomposed by the Kjeldahl method requiring but 7 c.c.  $n/4$  acid to neutralize, as against 19 c.c. required by the original serum. Analysis showed it to contain but 2.8 per cent of protein coagulable by alcohol, and this protein contained only 6.3 per cent of iodine. Evidently, therefore, a large proportion of this protein has been lost by precipitation, and the soluble remainder is but about one-half saturated with iodine—even less than Preparation I. The results of experiments with this preparation follow:

TABLE 9.

	First Injection	Interval	Second Injection	Results
1.....	0.02 c.c. bovine serum	32 days	5 c.c. iodized serum II	Death in 45 min.
2.....	0.02 iodized serum I	24 "	7 " "	Death in 45 min.
3.....	0.02 " " I	29 "	6 " " II	Death in 30 min.
4.....	0.02 " " I	53 "	5 " " II	Death in 55 min.
5.....	0.05 bovine serum	49 "	6 " " II	Severe symptoms; recovered
6.....	0.01 " "	19 "	4 " " II	Death in 55 min.
7.....	0.01 " "	19 "	3 " " II	Death in 140 min.

This preparation of iodized serum differs from the first preparation in the fact that it intoxicates guinea-pigs sensitized to normal serum equally as well as those sensitized to iodized serum.

*Iodized serum, Preparation III.*—On account of this disparity of results a third preparation of bovine serum was prepared, which resembled in all respects Preparation No. II. The heavy precipitate obtained from the serum was also collected and washed. It differed from iodized proteins generally in being but very slightly soluble in dilute (0.1 per cent) NaOH, and not at all in  $\text{Na}_2\text{CO}_3$  (1 per cent). Analysis of the insoluble residue showed it to contain 5.9 per cent of iodine, while the serum filtered from it, after dialysis, contained but 1.3 per cent of protein, and this protein contained but 1.88 per cent of iodine. Why these three preparations should differ so much from one another is not known, for all were prepared alike. The soluble part of the third preparation was found to have the same properties as Preparation II, and no further experiments were made along this line.

Rosenau and Anderson<sup>1</sup> in their latest publication make the following statement concerning the influence of iodine on serum, in

<sup>1</sup> *Jour. Med. Res.*, 1908, 19, p. 40.

respect to anaphylaxis: "We made several tests to determine the effect of iodine upon the toxic action of horse serum and it so turned out in the preliminary experiments that the symptoms appeared to be profoundly modified in the sense that they were either delayed or inhibited. We therefore tested a large number of guinea-pigs to determine this point but found that so far as the toxicity of horse serum is concerned at the second injection, it was not appreciably modified by the iodine. The iodine was added to horse serum in the proportion of 1.5 grams per twenty-five cubic centimeters of serum and three grams of potassium iodide to aid solution." Regarding the irregularity of the results obtained by Rosenau and Anderson, my own experiments show indications of similar irregularity, but in the case of iodized serum this is probably due to the incompleteness of the iodization as shown by analysis. The details of Rosenau and Anderson's experiments as published do not indicate that they controlled the completeness of their iodization by analysis.<sup>1</sup>

#### IODIZED EGG ALBUMIN.

Although such unreliable results were obtained with the iodization of serum, it was hoped that more satisfactory preparations might be obtained with pure proteins, and this was found to be the case. Using the pure egg albumin, recrystallized three times, and proceeding as with the iodization of the serum, an iodized protein was precipitated out in the dialyzing sac which seems to be a saturated iodized egg albumin. It contains 8.97 per cent of iodine,<sup>2</sup> agreeing well with Hofmeister's preparation of iodized egg albumin, which contained 8.9 per cent of iodine. It is quite insoluble in water, but dissolves slowly in 1 per cent  $\text{Na}_2\text{CO}_3$ , and readily in 0.1 per cent  $\text{NaOH}$ . The solution in dilute  $\text{NaOH}$  is not thrown down by  $\text{NaHCO}_3$ , but is by acetic acid, redissolving readily when the solution is made alkaline. The tyrosine is so well saturated that the protein does not give Millon's reaction, even when heated once to boiling; it does, however, give the Hopkins-Cole reaction for tryptophane. Therefore in all respects this preparation seems to be a typical saturated iodo-albumin.

In the experiments recorded below the iodo-protein was used in 5 per cent solution, dissolved in 0.1 per cent  $\text{NaOH}$ , to which was

<sup>1</sup> *Hygienic Laboratory Bulletin*, No. 45, 1908.

<sup>2</sup> For all the determinations of iodine I am indebted to Mr. Conrad Jacobson.

then added the calculated amount of  $\text{NaHCO}_3$  to convert all the sodium hydroxide into the less irritating sodium carbonate.

TABLE 10.

	First Injection	Interval	Second Injection	Results
1.....	0.001 gm. egg albumin	20 days	0.12 gm. iodized albumin	Severe symptoms
2.....	0.001 " "	18 "	0.15 " "	Death in 6 min.
3.....	0.1 " "	30 "	0.20 " "	Severe symptoms
4.....	0.1 " "	30 "	0.20 " "	Moderate symptoms
5.....	0.005 iodized alb.	23 "	0.15 " "	Slight symptoms
6.....	0.005 " "	23 "	0.15 " "	Slight symptoms
7.....	0.005 " "	23 "	0.15 " "	Severe symptoms
8.....	0.003 " "	23 "	0.13 " "	Death in 80 min.
9.....	0.0005 " "	23 "	0.13 " "	Death in 8 hours
10.....	0.0005 " "	26 "	0.20 egg albumin	No symptoms
11.....	0.005 " "	26 "	0.20 " "	No symptoms
12.....	0.005 " "	23 "	0.20 " "	Severe symptoms
13.....	0.005 " "	22 "	4.0 c.c. bovine serum	No symptoms
14.....	0.005 " "	22 "	5 dog serum	No symptoms
15.....	0.005 iodized serum I	17 "	0.15 gm. iodized alb.	No symptoms
16.....	0.05 " " I	21 "	0.15 " "	No symptoms
17.....	0.05 dog serum	17 "	0.15 " "	No symptoms
18.....	0.05 " "	21 "	0.15 " "	No symptoms

These experiments all indicate that the iodization of egg albumin and of serum does not have the same effect upon the specificity of the anaphylaxis reaction, that it was found to have upon the precipitin reaction by Obermayer and Pick. Iodized albumin does not sensitize to heterologous proteins, nor do heterologous proteins, even when iodized, sensitize to iodized egg albumin. Apparently iodized albumin does not effectively sensitize to the pure crystallized albumin, and it is not so effectively intoxicating to pigs sensitized with iodized protein as pure albumin is to pigs sensitized with egg albumin. Apparently, therefore, the saturation of the aromatic radicals with iodine decreases the toxicity of the proteins, and it also impairs their power of sensitizing animals to the original non-iodized protein. However, the somewhat impaired solubility of the iodized protein may here also play a part.

#### ACTION OF TRYPSIN UPON SERUM.

As one of the most characteristic features of proteins is their disintegration by proteolytic enzymes, and particularly as these enzymes affect nothing else than proteins, a very essential step in proving the protein nature of the bodies concerned in anaphylaxis is the determination of the effect of digesting solutions containing them with trypsin or pepsin or both. So far nothing positive has been



reported concerning this question, for the experiment of Rosenau and Anderson, who let serum stand over night at  $15^{\circ}$  with pancreatin, is of necessity devoid of significance, since under these conditions trypsin could have no appreciable effect upon the proteins of the serum. Unheated serum possesses such a resistance to the action of trypsin that even at  $38^{\circ}$  digestion proceeds very slowly for the first day, and any extensive disorganization of the serum proteins is a matter of months, as will be shown in the experiments to be cited below. In connection with their studies upon the chemistry of the specificity of proteins as shown by precipitin reactions, Obermayer and Pick investigated the effects of tryptic digestion upon serum and egg-white. They found that if the digestion was allowed to continue until the disappearance of the biuret reaction, the resulting material when injected into rabbits caused the development of precipitins which reacted only upon the same digestion mixture that was used in immunizing; this precipitin was, in spite of the proteolytic disintegration of the precipitogenous material, specific for the species furnishing the latter; i. e., precipitins produced by digested bovine serum will not react with digested horse serum, and conversely. Therefore, in spite of the extensive splitting of the proteins, the group upon which the specificity for species depends is intact even when the biuret grouping itself has been largely destroyed. Other experiments have indicated that digestion with pepsin-HCl causes the disappearance of precipitogens by the time all the coagulable proteins have disappeared, therefore much earlier than with tryptic digestion; also, a preparation obtained by digesting beef pancreas until biuret free did not produce precipitins.

Fleischmann,<sup>1</sup> who has also studied the effect of digestion upon the precipitin reaction, reaches results that are not in harmony with those obtained by Obermayer and Pick. He found that serum digested with trypsin loses its precipitability somewhat before the material ceases to give the usual protein reactions, while peptic digestion causes a similar condition in serum when but two-thirds of the coagulable protein has disappeared. He found that after about five months of tryptic digestion of a specimen of bovine serum it had lost its biuret reaction, and it was then used to immunize two rabbits.

<sup>1</sup> *Ztschr. f. klin. Med.*, 1906, 59, p. 515.

They were given 12 c.c. doses at intervals of 14 days, and one rabbit died after the second injection (probably it had been sensitized, for the conditions were ideal for such a result). The surviving rabbit showed no precipitin after the third injection, but after the fourth injection its serum contained a precipitin which precipitated heterologous proteins, such as goat and sheep serum and egg-white, and antibodies against human and rabbit serum were also demonstrated by fixation of complement. Evidently, therefore, sufficient digestion of a protein will destroy its specificity as indicated by the precipitin reaction. Fleischmann tabulates the results obtained by others who have investigated the effects of digestion upon the precipitogens of serum and finds marked discrepancies in the reports of different experimenters. These variations in results he ascribed to the variation in activity of different digestion mixtures.

#### EFFECT OF TRYPTIC DIGESTION UPON PROTEINS.

In connection with these results, and with those to be given below, should be considered the nature of the changes brought about in the protein molecule by the action upon it of trypsin. It has long been known that the process of proteolysis is not completed through a sudden collapse of the protein molecule, but rather it goes on step by step, with a gradual decrease in size of the molecule, so that at first we have the comparatively large proteose molecules and then the somewhat smaller peptone molecules, and finally the single amino-acid molecules are found free and the entire molecule of the protein has been converted into diffusible crystalloidal substances. The exact nature of these changes has been considerably cleared up of late by the investigations that are being carried on in the laboratory of Emil Fischer, and may be briefly summarized as follows:<sup>1</sup>

The first effect of the trypsin on the protein is to split out the tyrosine radicals, so that in a short time practically all the tyrosine is free in the digestion mixture, although the protein molecule is far from completely disintegrated. Tryptophane is also split out early, and likewise the cystine, while the other amino-acids hold together longer. For example, Abderhalden and Reinhold found that when edestin was digested with trypsin, after 24 hours 78.4 per cent of the tyrosine

<sup>1</sup> See Emil Abderhalden, *Lehrbuch der physiologischen Chemie*, Berlin, 1906, p. 179.

had been liberated, while of the glutamic acid but 4.3 per cent had been split out; after two days 97.6 per cent of the tyrosine as contrasted with 7.4 per cent of the glutamic acid had been liberated, and while all the tyrosine had been split out after 8 days, at this time but 31.1 per cent of the glutamic acid was free. Of the other amino-acids, alanine, leucine, amino-valerianic acid, and aspartic acid behave about the same as the glutamic acid. On the other hand, after very extensive digestion of proteins no free phenylalanine or proline can be found, for these radicals remain obstinately united, forming polypeptids which consist chiefly of these two amino-acids associated with small quantities of the other amino-acids exclusive of tyrosine, tryptophane, and cystine. If the protein contains glyco-coll this can commonly be demonstrated to resist cleavage along with the phenylalanine and proline.

It is evident, therefore, that the changes taking place in serum or other proteins undergoing tryptic digestion are such as tend to eliminate the tyrosine and tryptophane, leaving as the representative of the aromatic radicals the phenylalanine, which the gelatin experiments suggest is perhaps not a conspicuous factor in the intoxication of sensitized guinea-pigs. Fleischmann's observation that the specificity of proteins is impaired under the influence of protracted tryptic digestion, in which presumably most of the tyrosine and tryptophane have been removed, is in harmony with the claim of Obermayer and Pick that specificity depends upon the aromatic groups. However, the value of these observations is largely nullified by their lack of agreement, and also by the fact that one of the aromatic radicals, the phenylalanine, remains to the last with the residue of the protein molecules.

#### EFFECTS OF TRYPTIC DIGESTION OF BOVINE SERUM UPON ITS SENSITIZING AND INTOXICATING PROPERTIES.

The digestion of a specimen of bovine serum with trypsin was begun on February 28, 1908. To 750 c.c. of serum, which had been kept with a little chloroform for a few days in the refrigerator, was added 7.5 gm. of commercial pancreatin (Parke, Davis & Co.). This mixture was analyzed and found to contain per cubic centimeter 13.6 mg. of nitrogen, of which 90 per cent was coagulated by heat

and 10 per cent non-coagulable. There was then added 0.75 gm.  $\text{Na}_2\text{CO}_3$  and 10 c.c. chloroform, and the mixture was placed in a thermostat at  $37.5^\circ$ , being occasionally shaken.

After 10 days digestion (March 9) a sample was analyzed, and now but 22.7 per cent of the nitrogen was found to be coagulable, while 77.3 per cent was non-coagulable. The coagulum, after repeatedly washing with hot water, was found to still give both the Millon reaction for tyrosine and the Hopkins-Cole reaction for tryptophane, showing that these substances had not been entirely split out of the coagulable proteins. A part of the serum was set aside and labeled "Digestion Mixture I," and the remainder was returned to the incubator.

The results obtained with the digested serum<sup>1</sup> at this stage are shown in the following table:

TABLE 11.

	First Injection	Interval	Second Injection	Results
1....	6 c.c. digest. mixture	24 days	2 c.c. digestion mixture	Slight symptoms; recovered
2....	0.5 " "	25 "	3 " "	No symptoms
3....	0.1 " "	24 "	3 bovine serum	Death in 75 min.
4....	0.01 " "	24 "	2.5 " "	Very severe symptoms; recovered
5....	0.1 " "	24 "	2 digestion mixture	Slight symptoms; recovered
6....	0.01 " "	25 "	4.5 " "	Slight symptoms; recovered
7....	0.05 bovine serum	20 "	4 " "	Slight symptoms; recovered

The five pigs that recovered were each given 3 c.c. bovine serum on the next day, and showed slight or no symptoms, indicating that the second injection of digestion mixture, although not causing serious symptoms, had produced a refractory period such as follows recovery from intoxicating doses generally.

The results of this series of experiments may be summarized as follows:

After tryptic digestion of bovine serum until but 22.7 per cent of the nitrogen is in the form of coagulable proteins, the toxicity (intra-peritoneally) for sensitized pigs is almost entirely destroyed, although the sensitizing power for bovine serum remains, at least for sensitizing doses of 0.01 c.c. or more. Recovery from a second large dose of digestion mixture renders the animal refractory to bovine serum.

<sup>1</sup> In these and all other experiments in which material was used that had been preserved with chloroform, the material was freed from chloroform by placing in a shallow dish in a vacuum chamber for a short time before using it for injection.

After the digestion had continued 21 days there was a heavy precipitate in the mixture. A sample was filtered, and the filtrate analyzed. There had been some loss of nitrogen, presumably in part through precipitation and in part from escape of ammonia, for each cubic centimeter of filtrate contained 12.2 mg. nitrogen as against 14.6 mg. per cubic centimeter at the beginning. Of this nitrogen but 8 per cent was coagulable, and 92 per cent was not. The coagulum, however, still contained tyrosine and tryptophane as shown by appropriate tests. A portion of the digestion mixture, labeled "Digestion Mixture II," was reserved for experiments, and the remainder was returned to the incubator.

This preparation yielded the following results:

TABLE 12.

	First Injection		Interval	Second Injection		Results
1...	5	c.c. Digest. Mixt. II	21 days	5 c.c. bovine serum		Moderate symptoms; recovered
2...	0.25	" " "	21 "	5 Digestion Mixture		No symptoms
3...	0.25	" " "	21 "	5 horse serum		No symptoms
4...	0.04	" " "	21 "	5 bovine serum		Death in 35 min.
5...	0.04	" " "	21 "	10 milk		No symptoms
6...	0.004	" " "	21 "	5 bovine serum		Severe symptoms; recovered
7...	0.004	" " "	21 "	5 Digestion Mixture		No symptoms
8...	0.0004	" " "	34 "	5 bovine serum		No symptoms
9...	0.0004	" " "	21 "	5 " "		No symptoms
10...	0.05	bovine serum	18 "	5 Digestion Mixture		No symptoms
11...	0.05	" "	18 "	5 " "		No symptoms

Twelve days after the second injection a third injection was given each pig (except Nos. 4 and 8), this time consisting of 3 c.c. of bovine serum in each case. The results were as follows: (1) Second injection was bovine serum. Developed slight symptoms, but never became very sick; (2) Second injection was digestion mixture. Became very sick in 15 minutes and nearly died, but recovered eventually; (3) Second injection was horse serum. Died in 75 minutes; (5) Second injection was milk. Died in 70 minutes; (6) Second injection was bovine serum. Showed only slight symptoms; (7) Second injection was digestion mixture. Died in between two and four hours; (9) Second injection was bovine serum. Showed only slight or doubtful symptoms.

The results of this series may be summarized as follows: Digestion of serum by trypsin until but 8 per cent of the nitrogen exists as coagulable protein destroys, or at least greatly reduces the poisonous action on sensitized pigs, so that 5 c.c. intra-abdominally is without effect. This digestion mixture, however, sensitizes in doses of 0.004 c.c. but not in doses of 0.0004 c.c., as contrasted with normal serum which sensitizes uniformly in doses of 0.0001 c.c. and usually in doses of 0.00001 c.c. It does not intoxicate animals sensitized to

itself, or to unaltered serum, and the second large dose of digestion mixture does not make the animals refractory to bovine serum given 12 days later, although if the second dose is bovine serum the animals are refractory. The specificity of the serum has not been destroyed, for animals sensitized to digested bovine serum do not react to horse serum or milk, and are not rendered refractory by these substances.

After 59 days digestion another sample was removed for study, the remainder was filtered to remove the heavy precipitate, and 1 gm. pancreatin and 5 c.c. chloroform were added, and it was returned to the incubator. The sample, after filtration, contains 4.3 per cent of its nitrogen in coagulable forms and 95.7 per cent not coagulable and the coagulated protein still gives good reactions for tyrosine and tryptophane, the filtrate giving a strong biuret reaction. This material, designated as "Digestion Mixture III," was used in the following experiments:

TABLE 13.

	First Injection	Interval	Second Injection	Results
1...	0.1 c.c. Digest. Mixt. III	28 days	3 c.c. bovine serum	Death in 22 min.
2...	0.02 " " "	23 "	3 " "	Death in 60 min.
3...	0.01 " " "	23 "	3 " "	No symptoms
4...	0.005 " " "	23 "	3 " "	No symptoms
5...	0.002 " " "	28 "	3 " "	No definite symptoms
6...	0.001 " " "	28 "	4 " "	No definite symptoms
7...	0.0005 " " "	28 "	4 " "	No symptoms
8...	0.0002 " " "	28 "	4 " "	No symptoms
9...	1.0 " " "	28 "	6 Digest. Mixt. III	Death in 1 hour
10...	0.02 " " "	28 "	5 " " "	No definite symptoms
11...	0.16 " " "	22 "	5 " " "	Death in 2 hours
12...	0.05 bovine serum	16 "	5 " " "	No symptoms
13...	0.16 Digest. Mixt. III	21 "	0.2 gm. zein	No symptoms
14...	0.16 " " "	21 "	5 c.c. dog serum	No symptoms
15...	0.16 " " "	21 "	5 horse serum	No symptoms
16...	0.16 " " "	21 "	0.2 gm. egg albumin	No symptoms
17...	0.16 " " "	31 "	1 c.c. Digestion Mixture (intracardiac)	Death in 1 min.
18...	0.16 " " "	22 "	1 " " "	Severe symptoms; recovered
19...	0.16 " " "	22 "	1 " " "	Death in 4 min.
20...	0.01 bovine serum	28 "	1 " " "	Death in 4 min.
21...	0.1 " " "	35 "	1 " " "	No symptoms
22...	0.1 " " "	35 "	1 " " "	No symptoms
23...	0.05 gm. gliadin	30 "	1 " " "	No symptoms
24...	0.05 " " "	30 "	1 " " "	No symptoms
25...	0.05 zein	30 "	1 " " "	No symptoms

These experiments show that by tryptic digestion the sensitizing power of serum is steadily reduced, so that after 59 days digestion of this sample (when but 4.7 per cent of the nitrogen is coagulable), the minimum sensitizing dose to undigested serum is one-fiftieth of a cubic centimeter as contrasted with normal serum which sensitizes in doses of one-hundred-thousandth cubic centimeter or less.

On the other hand, the toxicity is so reduced that 5 c.c. intraperitoneally or 1 c.c. by intracardiac injection do not ordinarily produce symptoms in guinea-pigs sensitized to bovine serum. However, pigs sensitized to the digestion mixture died with typical symptoms when injected with 5 or 6 c.c. of the same intraperitoneally, and pigs with the same sensitization usually died when given 1 c.c. by the intracardiac route, whereas normal pigs treated in the same way showed no effects. Possibly this indicates a resemblance to some of the features of Obermayer and Pick's results on immunization with serum that had been digested with trypsin, for they found that it produced precipitins reacting with the digested serum but not with undigested serum. The extensive digestion of this experiment seems not to have at all impaired the specificity of the serum for it does not sensitize to zein, egg albumin, horse serum, or dog serum. Pigs sensitized with heterogenous proteins, and usually also even those injected with normal bovine serum, were not sensitized to the digestion mixture.

On July 19, 1908, after digestion for 129 days, this serum contained but 2.5 per cent of its nitrogen in coagulable form, while the biuret action is feeble, indicating that nearly all the protein has been decomposed into free amino-acids except a small portion which obstinately remains as a soluble protein, apparently possessed of a great resistance to trypsin. A sample of this material called "Digestion Mixture IV" gave the following results:

TABLE 14.

	First Injection	Interval	Second Injection	Results
1...	1.0 c.c. Digest. Mix. IV	25 days	4 c.c. bovine serum	Death in 45 min.
2...	0.1 " " "	25 "	4 " "	Death in 45 min.
3...	0.02 " " "	25 "	4 " "	Doubtful symptoms
4...	0.01 " " "	25 "	4 " "	No symptoms
5...	0.005 " " "	25 "	4 " "	No symptoms
6...	0.2 " " "	26 "	5 dog serum	No symptoms
7...	1.0 " " "	26 "	0.2 gm. egg albumin	No symptoms
8...	0.5 " " "	21 "	5.0 c.c. Digestion Mixture IV	No symptoms
9...	0.02 bovine serum	21 "	5 " "	No symptoms
10...	0.02 " "	21 "	5 " "	No symptoms
11...	0.02 " "	80 "	1 Digest. Mixt. IV (intracar.)	No symptoms
12...	0.02 " "	40 "	1 " " " "	Death in 4 min.
13...	0.5 Digest. Mix. IV	21 "	1 " " " (intracar.)	Death in 2 min.
14...	0.5 " " "	21 "	1 " " " "	Death in 3 min.
15...	0.5 " " "	21 "	1 " " " "	Death in 4 min.
16...	0.1 gm. egg albumin	60 "	1 " " " "	No symptoms

Evidently between the 59th and 129th days of digestion the only change has been a slight reduction in the sensitizing power so that

$\frac{1}{50}$  c.c. scarcely produces effects, while  $\frac{1}{10}$  c.c. sensitizes fatally. This latter preparation also fails to sensitize to heterologous proteins (dog serum and egg albumin), and these heterologous proteins do not render the animals refractory to a dose of bovine serum given 24 hours later. The digestion of this serum is being continued and the eventual changes will be studied and reported. Similar studies with pure crystallized egg albumin are also under way.

#### MISCELLANEOUS OBSERVATIONS.

A few miscellaneous observations have been made in the course of these experiments, which, although not bearing directly upon the problems in hand, should probably be included in this report.

One concerns the mutual sensitizing and intoxicating power of bovine serum and bovine milk. As both serum and milk in this case come from the same species, it is of interest to consider their relation to one another in respect to anaphylaxis. The following experiments seem to indicate that this relation is a very uncertain one, the one fatality standing alone.

TABLE 15.

	First Injection	Interval	Second Injection	Results
1.....	0.02 c.c. bovine serum	40 days	5 c.c. milk	Slight symptoms
2.....	0.02 " "	57 "	10 "	Died in 110 min.
3.....	0.02 " "	46 "	10 "	No symptoms
4.....	0.02 " "	40 "	10 "	No symptoms
5.....	0.02 " "	46 "	10 "	No symptoms
6.....	1 milk	10 "	5 bovine serum	Slight symptoms
7.....	1 "	10 "	5 "	Slight symptoms

Animals recovering from milk after bovine sensitization (Nos. 1, 3, 4, and 5) were found to be fully sensitive to bovine serum when this was injected 24 hours later, showing that the milk had not rendered them refractory to serum.

The second is an observation upon the effect of preservation upon horse serum. Rosenau and Anderson state that preservation of horse serum for  $8\frac{1}{2}$  years does not appreciably alter its intoxicating effect. A specimen of horse serum that had been preserved for about fifteen months in an ordinary refrigerator, with the use of chloroform in the serum, was kindly furnished me by Dr. N. MacL. Harris, and in the course of certain studies the following observations were made:



TABLE 16.

	First Injection	Interval	Second Injection	Results
1...	0.01 c.c. old horse serum	18 days	5 c.c. bovine serum	Death in 2 hours, with typical symptoms
2...	0.1 " " "	18 "	5 " "	Death after 6 hours, following typical symptoms
3...	1.0 " " "	22 "	2 egg white	Moderate but typical symptoms. 24 hrs. later given 6 c.c. bovine serum and showed slight symptoms
4...	0.05 " " "	36 "	5 bovine serum	Moderate symptoms
5...	0.05 " " "	36 "	5 " "	Very slight symptoms
6...	0.05 " " "	36 "	5 dog serum	Slight symptoms
7...	0.05 " " "	36 "	1.5 egg-white	Very severe symptoms
8...	0.05 " " "	36 "	1.5 " "	Very slight symptoms
9...	0.05 " " "	36 "	10 milk	No symptoms
10...	0.05 " " "	53 "	5 bovine serum	Moderate symptoms

All these pigs that recovered were given later 3 c.c. of horse serum each, which caused in each case more or less severe symptoms but none of the animals died. These experiments suggest that long preservation of serum under chloroform lessens somewhat its specificity as a sensitizing agent, for the degree of intoxication observed in several of these pigs was much more marked than is usual in experiments with heterologous proteins, and they are cited for what they are worth. As they do not immediately concern the work in hand, the suggestion that they offer has not been followed up. It may be mentioned that Obermayer and Pick noted that long standing under toluol alters serum in such a way that it produces precipitins that are at least quantitatively specific for the same toluol-altered serum, but the species specificity it preserved. In the above experiments the species specificity seems to be somewhat impaired, whether by the long action of the chloroform used in preservation, or by slow auto-digestion, or some other change, is not apparent.

## SUMMARY.

Egg albumin, freed from the other proteins of egg-white by repeated crystallization, produces typically the anaphylaxis reaction. It sensitizes in doses as small as one-twenty-millionth of a gram; fatally in doses of one-millionth of a gram. The minimum lethal dose for sensitized pigs is about one-half a milligram by intraperitoneal injection, and about one-tenth to one-twentieth of a milligram when injected into the circulation.

The unpurified mixed proteins of egg-white are much less active than the pure albumin, the minimum sensitizing dose being about

one hundred times greater and the minimum lethal dose being five times greater than with purified egg albumin. This suggests the possible presence of inhibiting substances in crude egg-white.

So small is the quantity of egg albumin that is fatal to sensitized pigs, one-tenth of a milligram, or less, that it seems improbable that the injected protein itself can cause death by splitting up and liberating poisonous substances in the space of time in which this reaction occurs. More probably if poisonous substances are produced, they must be produced by the cells of the intoxicated animal from some of the substances in its own body, or the whole reaction may be essentially different from any ordinary intoxication.

The minuteness of the minimum sensitizing and intoxicating doses of pure egg albumin seems to indicate conclusively that, at least in the case of this particular protein, both the sensitizing and the intoxicating agent are one and the same kind of protein molecule or else two different portions of the same molecule.

Gelatin seems to be devoid of the power of participating in the anaphylaxis reaction, either with itself or with other proteins. This may be due to its poverty in aromatic radicals; it probably is not due to the heating that is necessary for the conversion of collagen into gelatin. Addition of tyrosin to gelatin (without chemical combination) does not modify gelatin in respect to the anaphylaxis reaction.

Milk does not lose its sensitizing or intoxicating power when heated to 100° for 30 minutes. If large enough doses of serum heated to the same degree are used they will sensitize guinea-pigs to serum.

Coagulation with alcohol destroys or reduces greatly the toxicity of proteins which it renders insoluble in water (egg albumin) but not of proteins which it does not render insoluble (serum albumin).

Crystallized egg albumin does not entirely lose its sensitizing power when heated in aqueous solution to 100° for 15 minutes, if large doses are used. Heating to 90°, however, nearly destroys its intoxicating effect. Increasing the coarseness of the coagulated particles by addition of acetic acid seems to somewhat increase the effect of heating. Probably the destruction or reduction of toxicity of proteins by heat is due to the change in solubility of the proteins.

Pure zein is actively and specifically toxic to guinea-pigs sensitized with zein, although this protein is devoid of tryptophane and lysine.

Gliadin, which contains a less quantity of aromatic radicals than almost any other protein except gelatin, has but slight power to intoxicate pigs previously injected with gliadin.

Iodization of different specimens of serum by a constant method did not yield constant results. The partially saturated serum proteins did not suffer any modification in specificity analogous to that found by Obermayer and Pick in the case of the precipitin reaction. When most nearly saturated they may lose the power of sensitizing for the unaltered serum, but this is uncertain.

Pure crystallized egg albumin may be saturated with iodine quite readily, the iodine saturating the unsaturated carbon atoms of the benzene ring. Such iodized albumin retains its specificity unaltered, but seems to lose much of its toxicity for sensitized guinea-pigs, and its power to sensitize to egg albumin is impaired.

Tryptic digestion of serum furnishes further evidence of the protein nature of the substances concerned in the anaphylaxis reaction. Both sensitizing and intoxicating principles are attacked, and slowly decrease in strength as the coagulable protein disappears. After 59 days digestion of a sample of serum, so that but 4.7 per cent of the nitrogen was still in a coagulable form, the sensitizing dose had been changed from one-thousandth of a cubic centimeter to one-fiftieth of a cubic centimeter, while 5 c.c. intraperitoneally did not intoxicate pigs previously sensitized to bovine serum. Digestion of serum to this extent does not affect its specificity for species, but the digested serum sensitizes much better to itself than to undigested bovine serum, and conversely, it intoxicates pigs sensitized to itself much more than it intoxicates pigs sensitized to unaltered bovine serum.

Possibly the specificity of horse serum suffers somewhat after prolonged preservation with chloroform.



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## PLAGUE AMONG THE GROUND SQUIRRELS OF CALIFORNIA.\*

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THE fact that a number of ground squirrels have been proven to be infected with *Bacillus pestis* in two widely separated sections of the state of California is perhaps the most serious feature of the plague situation in America. Ground squirrels of various and more or less closely related species may truly be said to be innumerable throughout the mountains and lowlands of the western states from Alaska to and beyond the Mexican boundary line. Hillsides, railroad cuts, river banks, and fields are literally perforated by their complicated systems of subterranean tunnels and innumerable inlets and outlets. Ranchers pray for the day when some efficient means for their extermination is found and their ravages cease. Of all the places in the earth "North America is richest in the *Arctomyiinae*, her ground squirrels being numerous and diversified." Represented by the various rock squirrels of the Rockies, the prairie dogs of the western plains, and the groundhogs of the eastern states they reach from the Atlantic to the Pacific Ocean.

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## THE OCCURRENCE OF PLAGUE AMONG THE ARCTOMYINAE AND SCIURINAE ELSEWHERE IN THE WORLD.

"While tree squirrels (*Sciurinae*) abound in the torrid as well as in the temperate zone, the ground squirrels (*Arctomyinac*) are more northerly, on the whole being confined to temperate and colder latitudes, and next to America occur in greatest numbers in Asia."

Extending between 50° and 54° N. throughout the transbaikal region of Eastern Siberia and Northern Mongolia there exists a rodent larger than, but closely related to, our eastern "groundhog." The Mongols call them "tarbagans." In Russian they are called "beibak" and in German "Bobak" (*Arctomys bobac*, Schreb.). According to Rudenko<sup>1</sup> these animals dig long passages under the ground where they have their dens. They hibernate for seven months, usually from September 14 to March 15, and when they emerge in the spring they are much hunted for the sake of their flesh and fat. There are years when the tarbagans are swept by an epizootic which ceases by the end of summer. The sick animals will not go into their burrows, they are sleepy, crawl slowly, or stagger while running. When examined, a tense red swelling may be seen under the shoulder and this exudes dark fluid blood on section. They appear to be harmless to dogs when eaten and yet the Burjats, Cossacks, and Mongols fear these sick animals, as apparently their sickness may be transmitted to man, who then dies quickly. There is no reasonable doubt that this disease of tarbagans is plague, as Rudenko contends. One of Rudenko's accounts of human infection following the handling of sick tarbagans is particularly interesting:

In September, 1894, a number of deaths occurred in the family of the Cossack Mirsanoff, in the village of Sektuevsk. It seems that the father Mirsanoff had to go out of his village to Zaganolin to attend court. His pet dog trotting along beside him managed to kill six tarbagans in a very short time. Now the fact that the dog could kill six tarbagans while running by his master's side indicates that the animals must have been sick; healthy tarbagans are never caught under such conditions by a dog. Mirsanoff took the tarbagans along with him and later hid them in a hay pile in order to take them home on his way back; which he did on August 31. On September 2, he became ill and died on the 5th. As the illness was thought to be due to catching cold, no preventive measures were taken. In spite of the customary burial with a wake, none of the guests became ill. But six days later one of the sons became ill and died two days later, with manifest swelling of the glands in the axillary and

<sup>1</sup> *Russian Military Med. Jour.*, 1900, pp. 35, 67, reviewed by L. Heydenreich, "Die Pest der Tarbaganen," *Centralbl. f. Bakt.*, 1901, 29, p. 218.

inguinal regions. Now the family and neighbors recognized the danger. The family were strictly isolated in a vacant house, but a day or so later (September 15) two of the sons went into the forest after some wood and returned on the 18th, but one was brought home dead of the same malady. A daughter became ill on the 17th and died on the 21st. On the 19th still another son became ill and died on the 22d. The 80-year-old grandmother became ill on the 20th and died on the same day. On October 2 another daughter fell ill, became delirious, and died on the 3d.

And again according to Bannerman,<sup>1</sup> the common striped squirrel, *Sciurus palmarum*, so numerous in the verandahs and gardens of India, has been found to suffer from epidemic seizures of plague.

In December, 1898, a grey-striped squirrel was picked up dead at Cadag, in the southern portion of the Bombay presidency, where plague was at the time prevalent. There was no bubo in this animal but the spleen was enlarged and plague bacilli were isolated from its tissues.<sup>2</sup>

#### REVIEW OF THE EVENTS WHICH LED TO THE DISCOVERY OF PLAGUE AMONG SQUIRRELS IN CALIFORNIA.

I am greatly indebted to the generosity of P. A. Surgeon Rupert Blue, commanding plague-suppressive measures in San Francisco and vicinity, for data in this section, and I shall quote from his report. However, I believe it will be well to introduce some additional data which, along with reference to the accompanying relief map, will enable a reader unacquainted with local conditions to gain a proper orientation.

It will be remembered that, during the present pandemic of plague, the disease was first detected in San Francisco in 1900, when there were 22 fatal cases. These were practically limited to the district enclosing Chinatown. In 1901 there were 30 cases with 25 deaths in San Francisco. In 1902, 41 fatal cases and, in 1903, 17 fatal cases were reported from San Francisco. Now Dr. Blue reports:

While investigating the origin of one of the early cases of plague in San Francisco (Bock, 1903) I was impressed with the possibility of ground-squirrel infection in Contra Costa County. The efforts made at that time, however, to prove the hypothesis by finding an infected squirrel failed. Physicians and ranchers living in that section of the state, while apparently deeply interested in the subject, could not or would not devote sufficient time to the investigation. Two specimens only were obtained and in these putrefaction was too far advanced to admit of a bacteriological examination.

In 1903 it was rumored that an epizootic had destroyed the ground squirrels of the upper and middle portions of the county the year before. The report was con-

<sup>1</sup> *Jour. of Hyg.*, 1906, 6, p. 182.

<sup>2</sup> Dr. Alice Corthorn, "Plague in Monkeys and Squirrels," *Indian Med. Gazette*, March, 1899; referred to as above quoted by F. G. Clemow, "Remarks on Plague in the Lower Animals," *Brit. Med. Jour.*, 1900, 1, p. 1141.



MAP.—The stars in the region of Bay Point, which projects northward into Suisun Bay, indicate the localities where plague-infected squirrels were found. The star with a white center indicates the location of the Faria Ranch, where a boy, J. F., died of plague on July 15, and where a squirrel dead of plague was found, on August 5.



firmed by several inspections which were made with the view of defining the boundaries of the infected area, and to obtain sick or dead squirrels. About this time (August, 1903) a country blacksmith, Charles Bock by name, of Pacheco, Contra Costa County, came to San Francisco and died of plague in the German Hospital. In order to investigate his history I visited Pacheco, Cal., and while there was informed that Bock had shot ground squirrels three or four days before his illness began. He had not in the past thirty days visited Oakland or San Francisco.

On September 13, 1903, E. T. S., a bridge-builder, employed by the Southern Pacific Company, in construction work near Danville, Contra Costa County, died of bubo-septicemic plague, in the Southern Pacific Hospital in San Francisco. He had lived for the past two months in a railroad camp in the San Ramon Valley, thirty miles away from the centers of population. Ground squirrels had often been killed and eaten as a delicacy by the rough laborers.

In 1904, nine cases of plague with eight deaths were recorded in San Francisco. With regard to the occurrence of cases outside of San Francisco, Dr. Blue reports:

In February, 1904, Dr. F. F. Neff, a practicing physician of Contra Costa County, reported a suspicious case in a woman living near Concord, Cal. P. A. Surgeon B. J. Lloyd, then on duty at the Plague Laboratory, Chinatown, was detailed to investigate the case. He reported that the patient, Mrs. F. S., had died of bubo-septicemic plague soon after his arrival at her home. Some of the glands were excised and examined at the plague laboratory and showed typical gross and microscopical findings of plague.

P. A. Surgeon Donald Currie, who was then bacteriologist for the plague laboratory, carried on a series of experiments with a view to ascertaining the susceptibility of the ground squirrel to plague infection, by inoculation and direct contact. This work was done in the summer of 1904 and gave positive results in both cases. Some three dozen squirrels were used in the experiments. The animals were obtained through an advertisement inserted in a daily paper, being trapped in San Mateo County.

In September, 1904, a thorough inspection was made of Contra Costa County, for the purpose of locating accurately the sections covered by the rodent epizootic of 1902-3. Dr. Medros, acting under my direction, spent a week driving through the country and interviewing the farmers in regard to the disappearance of rodents. He ascertained that the epizootic began on the water front at Port Costa in 1903, and extended southward to Walnut Creek and eastward to Antioch in a year. In this triangular section the squirrels had almost entirely disappeared. Only a few of the people interviewed had seen sick or dead animals, but all had noticed the rapidly diminishing numbers. It was generally believed that the mortality was due to a "contagious disease" which had been started among them by the professors of the University of California. The experiments of Dr. Currie, however, disproved this theory, as squirrels are not susceptible to infection by *Danysz*, or to any of the viruses in common use.

The authorities at the State University denied that any such attempt to exterminate squirrels had been undertaken by any of their staff, when inquiries were made by Passed Assistant Surgeon J. D. Long and myself.

Passed Assistant Surgeon J. D. Long, until recently in charge of the situation in Alameda and Contra Costa counties, made inquiries concerning past epizootics among the ground squirrels and learned from Mr. Hook, a supervisor of Contra Costa County, that in the region of Martinez, between 1903 and 1905, on several occasions, great numbers of squirrels were seen to migrate across the country, so that in places where they had been numerous they became scarce and vice versa; and in the neighborhood of Livermore, Alameda County, a rancher who appeared to be a keen observer had given similar accounts and had added that persons came into the region where sick squirrels were dying, and had taken sick squirrels elsewhere in order that the disease might spread. At one time Dr. N. K. Foster, secretary of the State Board of Health, offered as much as \$20.00 for a sick squirrel but was unable to obtain one.

In 1905 and 1906 no cases of plague were observed in San Francisco. Dr. Blue further reports on the status of affairs in Contra Costa County:

There were no suspicious cases reported in the summer of 1904, or in 1905, although a careful watch had been maintained by Dr. Neff and others in the county. In March of 1906, however, a boy sickened in East Oakland with typical bubonic plague. The investigation which followed failed to connect the boy with a previous case of human plague, but showed that he had shot and eaten ground squirrels three or four days prior to his seizure. The squirrels were killed in Strawberry Canyon, which is about half a mile east of the grounds of the University of California.

In 1907, after the earthquake, plague again appeared in San Francisco. The first case detected was in a sailor, from the tugboat "Wizard," who died at the Marine Hospital at San Francisco, and was diagnosed by Passed Assistant Surgeon J. D. Long. During the year 156 cases with 78 deaths occurred in San Francisco. Ten cases were reported from Oakland. The last case of human plague in San Francisco was on January 30, 1908, and the last infected rat found in that city was found on July 28, 1908. From all over the city 30,880 rats have been examined since that time, with negative results. The last case of human plague (excepting one) in Oakland was found on December 22. A number of infected rats were found in this city in the spring months, but none between April 15 and July 15 (however, only 9,361 rats were examined). For the history of

the last case found in Oakland (July 21, 1908) I would refer the reader to the last section of this article.

Now, a boy, J. F., died July 15 on a ranch out of Concord, Contra Costa County (see star with white center on map). He was reported as suspicious by Dr. DeWitt, county health officer, and autopsied by Dr. W. H. Harrison, Jr., state inspector. It was subsequently proven by laboratory methods that the boy had died of plague. And on July 28, another case (M. P.) was found in this county, in Briones Valley, about 10 miles from Martinez. Investigations by Dr. Foster, Dr. Long, Dr. Rucker, and Dr. Howe revealed the fact that there had been a mortality among the rats on a ranch near the place where the boy, J. F., died. Here a rat was found dead and turned over to Passed Assistant Surgeon McCoy, who found that it was anatomically suspicious for plague, but decomposition was too far advanced to prove it to be plague by animal inoculation. Dr. Blue directed that men be sent from Dr. Long's force in Oakland to trap and collect rodents in Contra Costa County. Between August 5 and October 10, 423 ground squirrels, 82 rats, 3 mice, 18 jack-rabbits, 2 chickens, 1 gopher, 1 ground owl, and 1 coyote have been received for examination at the Oakland Plague Laboratory of the U. S. P. H. and M. H. S. Most of these animals were shot. Four out of the 423 ground squirrels were infected with *B. pestis*, as detailed in the next section of this article. Three of the four were found dead. Number 1 was found dead on August 5, near a barn on the Faria ranch, where the boy, J. F., died on July 15. No other evidence of any disease which might explain the histories of past epizootics among the animals in this region was obtained.

The results of Dr. Currie's experimental work with ground squirrels combined with the epidemiological data accumulated convinced many that the ground squirrels were a not infrequent source of human infection. It is only fair to state here that I, personally, was skeptical of its importance. However, the history of the case which occurred in Los Angeles, combined with the observations of Dr. Currie that a considerable percentage of inoculated squirrels develop plague pneumonia, and the fact that the mucus in the posterior nares of squirrel No. 1 (*vide infra*) was proven to be infectious, leaves no room for doubt that the bite of a plague-sick squirrel may result in

infection. For the history of the Los Angeles case I am indebted to Passed Assistant Surgeon Geo. W. McCoy, who was detailed to investigate the case. He reports as follows:

I arrived in Los Angeles on August 30, 1908. Surgeon Brooks, Public Health and Marine-Hospital Service, and Dr. Powers, the health officer, met me at the train and after a short preliminary conference at Dr. Brooks's office, we visited the case. The patient was a boy ten years old, who lived with his parents at Elysian Park in the northeastern part of the city, about two miles from the city hall. This park adjoins the yards of the San Francisco-Los Angeles line of the Southern Pacific Railroad.

Several days prior to the onset of his illness, the boy found a ground squirrel near his home. The animal appeared sick and made no effort to escape from the boy when he picked it up, but it bit him on one of the fingers of the right hand. Just what became of this squirrel is uncertain. It is believed to have been killed by a cat or a dog. Four or five days later, on August 11, the boy became ill with a headache, delirium, and fever which rapidly rose to 106° F. and for several days he was quite sick. On the second or third day of the illness the right axillary glands became swollen and painful and later the other superficial glands became enlarged.

The boy was under the professional care of Dr. E. B. Alexander. Dr. Alexander called Health Officer Powers to see the case and later Surgeon Brooks was asked to see it. All of these gentlemen had had experience with plague and all regarded this case as decidedly suspicious.

On August 20, the right axillary gland was aspirated and a few drops of bloody fluid obtained. Nothing was to be seen in the smears, but a culture was made in broth which gave a growth that was regarded as a streptococcus, but from the appearance of the stained specimens from this culture submitted to me for examination I am convinced that the organism was a bacillus growing in chains. This culture was turned over to me and after making a careful study of its growth on artificial media and conducting inoculation experiments on animals (rats and guinea-pigs) I have demonstrated that the organism is *B. pestis*. When I saw the boy on August 30, he was without fever and appeared to be convalescent, but was very weak. The right femoral gland had been incised and there was a free discharge of pus. The right axillary glands were much enlarged. There was great infiltration of the surrounding tissue and softening beginning at one point. The history, with the appearance of the case at the time I saw it, compelled me to share the suspicions of those who had seen it earlier.

On August 21 a squirrel was found dead within 50 yards of the place where the boy had been bitten. There were no signs of injury and a post-mortem examination by Dr. Black, professor of bacteriology in the local medical college, showed an inguinal bubo and large spleen. This bubo and the spleen were used to inoculate a guinea-pig by the cutaneous method. The guinea-pig was found dead on the morning of August 28. The guinea-pig had been kept in Kaiserling's solution for my examination. It presented a caseous bubo, a dark liver with many whitish granules, and a large spleen full of whitish granules, in other words, the typical appearance of plague in the guinea-pig. Smears made from the liver and spleen by Dr. Black showed an abundance of bipolar bacilli and coccoid forms. A culture was made from the heart's blood. This culture was turned over to me for study and it answers all of the requirements for *B. pestis*. It should especially be mentioned that it gives charac-

teristic growth on agar and in broth and gives characteristic involution forms in 24 hours on salt agar. White rats, wild rats (*Mus norvegicus*), and guinea-pigs were inoculated with this culture, and all reacted with the typical lesions of plague.

I made post-mortem examinations of several squirrels and two cats, all killed or found dead in the vicinity where the dead squirrel was found. All were negative for plague.

#### THE ANATOMIC AND BACTERIOLOGIC FEATURES OF SQUIRREL PLAGUE AS OBSERVED IN CALIFORNIA.

All of the ground squirrels examined up to date have been of one species, *Otospermophilus beecheyi* (Richardson).<sup>1</sup>

*Squirrel No. 1.*—Found dead on the Faria Ranch, and received for examination on August 6, 1908.

On dissection the subcutaneous tissues showed some injection; the spleen and liver were somewhat enlarged, dark, and soft; there was a small amount of clear fluid in the thoracic cavity; there were areas of consolidation in the lungs. Upon the removal of thoracic organs *in toto* the anterior lobes of both lungs showed grayish areas of consolidation, involving the whole anterior lobe and anterior portion of the posterior lobe on the left side and the anterior and middle lobes and part of the posterior lobe on the right side. The grayish areas were surrounded by deeply congested zones and without the areas of pneumonia there were quite a number of subpleural petechiae. The involved tissues appeared grayish red on section, and exuded an anchovy-sauce-like material upon pressure. (See Plate 4, Fig. 1.)

<sup>1</sup> The following description of this species is given by E. A. Mearns, M.D., major and surgeon U. S. Army, in "Mammals of the Mexican Boundary of the United States," *Bull. 56* (1907) *U. S. National Museum*, pp. 314 ff.

#### *Otospermophilus beecheyi* (Richardson) CALIFORNIA GROUND SQUIRREL

Type-locality.—The neighborhood of San Francisco and Monterey, in California.

*Description.*—Size smaller than *Otospermophilus grammurus* (nearly as large as the eastern gray squirrel) with a more slender body and shorter tail. Ears high and pointed. Mammary glands, six pairs (P. 2/2, A. 2/2, I. 2/2=12). Color above brown, grizzled and annulated with black in a vermicular pattern; darkest anteriorly, and most grizzled and vermiculated posteriorly. Nape and sides of neck silver gray, this color prolonged backward above the shoulder in the form of stripes which are sometimes faintly traceable to the root of the tail, though usually ending about the middle of the body. Ears black outside, grayish, or faintly rusty inside, and along posterior border. Top of head bistre, slightly dusky above orbits, which are encircled by white. Sides of head grayish, mixed with yellowish brown. The tail, which is less bushy and shorter than in *O. grammurus*, is yellowish gray, the lateral hairs thrice annulated with black. Feet yellowish gray. Under surface of body grayish white. The inter-scapular region is often blackish, more or less vermiculated with pale annuli. Length, 410 mm.; tail vertebrae, 170; hind foot, 55; ear above crown, 21; ear above notch, 27; length of head, 62; skull, 57 by 34 mm. (averages).

Major Mearns observed that young were being born during the whole period of collection from May 10 to July 13, 1894. In coloration they closely resembled their parents at the same season. I have obtained some specimens larger than those obtained by Major Mearns.

Further examination revealed an ulcer, about 10 mm. in diameter on the back, just anterior to the root of the tail. On section the tissues beneath the ulcer showed congestion. This subcutaneous congestion was traced to an abscess about  $15 \times 10 \times 10$  mm. apparently occupying the position of the left inguinal glands. The abscess was full of a pale greenish-yellow, viscous, purulent material.

*Microscopic examinations.*—Smears from the consolidated areas in the lung showed enormous numbers of bipolar-staining rods and coccoid forms resembling *B. pestis* (Carbol Thionin). These were also very numerous in the mucus of the posterior nares and in the subcutaneous tissues just beneath the cutaneous ulcer. Numerous bipolar-staining bacilli resembling *B. pestis* were seen in smears of the pus from the inguinal abscess. Smears from the spleen and heart's blood showed only a few rods and these did not present a good bipolar appearance. Micrometric determinations (Zeiss, oil imm., oc. microm. 6) showed that the bipolar-staining rods in the lung smears varied from  $1.4\mu \times 1.2\mu$  up to  $1.6\mu \times 1.2\mu$  while the coccoid forms were generally  $1\mu$ – $1.2\mu$  in diameter. Comparative measurements made at the same time showed that the bipolar-rod forms of the bacillus of rabbit septicemia were  $1\mu \times 0.5\mu$  and the coccoid forms  $0.4\mu$ – $0.6\mu$  in diameter.

*Animal inoculations.*—A white rat was inoculated cutaneously by rubbing a piece of the consolidated pulmonary tissue into a scratch on its abdomen. It died in a little less than three days, showing marked subcutaneous congestion, hydrothorax and congestion of all the organs. Bacilli resembling *B. pestis* were few in the inguinal glands, but very numerous in the spleen, and were obtained in pure culture.

Guinea-pigs Nos. 1 and 2, inoculated cutaneously with pulmonary tissue, died after eight and seven days respectively, and showed marked subcutaneous injection, large hemorrhagic inguinal buboes, hydrothorax and multiple ecchymoses beneath the serosa of the lungs and large intestines. There were numerous bipolar-staining rods resembling *B. pestis* in preparations from the buboes and spleens. Cultures resembling plague were obtained from the spleen and heart's blood of each in purity.

Guinea-pig No. 3, inoculated cutaneously with a little of the mucus from the squirrel's posterior nares, died in seven days with the same anatomical changes as in guinea-pigs Nos. 1 and 2. Pure cultures resembling plague were isolated from its spleen and heart's blood.

Guinea-pig No. 4, inoculated cutaneously with purulent material from the inguinal abscess, died in five days with the above-described anatomical changes. Pure cultures resembling *B. pestis* were isolated from its spleen.

*Cultural studies.*—+1 agar slants were inoculated from the lung, spleen, and heart's blood of the squirrel. In 24 hours at  $37^{\circ}$  C. these showed numerous minute transparent colonies which were viscous. These growths yielded typical large club-shaped and yeast-like involution forms on 3 per cent salt agar after 24 hours' growth at  $37^{\circ}$  C.

The cultural characters of the bacilli isolated from the lung of the squirrel itself and from the spleens of white rat No. 1, and guinea-pigs Nos. 1, 2, 3, and 4 may be summed up as follows: They corresponded with *B. pestis* in that growths on +1 agar slants resembled those of *B. pestis*, and were viscous; they all formed large clubbed and yeast-like involution forms on +1.3 per cent salt agar in 24 hours at  $37^{\circ}$  C.; they grew as a stringy, viscous mass at the bottom of the test-tube in +1 sugar free bouillon;

they produced no visible change in + 1 milk: they grew delicately in and did not liquefy + 1.5 gelatin at 18-22° C.; they produced acid but no gas in + 1 broth containing 1 per cent of dextrose, levulose, galactose, maltose, and mannite, but no acid nor gas from lactose, saccharose, nor inulin. The culture from the heart's blood of white rat<sup>1</sup> No. 1 produced typical stalactites in + 1 oiled broth.

A piece of the squirrel's lung was sent to Passed Assistant Surgeon Geo. W. McCoy for confirmatory diagnosis. His report has already been published.<sup>1</sup> McCoy carried out a series of valuable experiments, some of which I quote:

While no doubt was entertained as to the nature of the organism under investigation, it seemed desirable to test it against antipest serum. For this purpose we used guinea-pigs and rats (*Mus norvegicus*). The culture was the same as in the above experiment (direct from the squirrel's lung), but was the third generation, and the growth was 48 hours old. In each case the animal was given subcutaneously 0.01 of a loopful of the agar culture. Three guinea-pigs were used.

The first guinea-pig was given 5 c.c. of antipest serum intraperitoneally just prior to the inoculation. This animal never sickened, and is alive and well at the present time, 10 days after the inoculation.

The second guinea-pig was given 5 c.c. of antidiphtheria serum intraperitoneally just prior to inoculation. This was done to provide a serum control. This animal died on the fourth day with characteristic lesions of plague, and cultures from the liver gave a pure culture of *Bacillus pestis*.

The third guinea-pig was inoculated with the culture in the same manner as the previous ones, but no serum was given; therefore it served as a control. This guinea-pig died on the fourth day and presented lesions characteristic of plague. An organism agreeing with the one isolated directly from the squirrel was obtained in pure culture from its liver.

Four rats (*Mus norvegicus*) were inoculated with the same amount of the same culture as was given to the guinea-pigs. Two of the rats that had previously been given 5 c.c. of antipest serum were killed on the eighth day and were shown by post-mortem examination to be normal. The two that had not received serum (controls) died on the fourth day and both presented typical lesions of plague as seen in the rats, and a pure culture of *Bacillus pestis* was isolated from the liver in each case.

In order to determine whether the organism was as virulent as the *Bacillus pestis* isolated from rats here, two series of guinea-pigs were inoculated with the results shown in the table below. An agar culture was used in each case.

QUALITY OF CULTURE	GUINEA-PIG DIED	
	Squirrel	Rat No. 66
	Second Generation	(Fourth Generation)
1 loopful, vaccinated .....	Fourth day	Fifth day
0.01 loopful, inoculated, subcutaneously .....	Fifth "	Third "
0.001 loopful, inoculated, subcutaneously .....	" "	Seventh "
0.0001 loopful, inoculated, subcutaneously .....	Eighth "	Sixth "
0.00001 loopful, inoculated, subcutaneously .....	Fifth "	Seventh "
0.000001 loopful, inoculated, subcutaneously .....	Seventh "	Sixteenth "

<sup>1</sup> *Public Health Reports*, 1908, 23, p. 1289.

The control was *Bacillus pestis*, isolated from plague rat No. 66. Neither of these cultures had ever been through an animal other than the ones in which they were found in nature. It will be observed that there is no material difference in the virulence of the cultures.

*Squirrel No. 2.*—Found dead on the Morton ranch, Contra Costa County, on August 21, 1908. Received for examination August 22.

The body showed marked post-mortem decomposition and was badly fly-blown. There was an abscess  $8 \times 5 \times 5$  mm. in the right inguinal region and what remained of the left axillary gland indicated that it might have been caseous. The lungs were badly macerated but a small area was found which was of firmer consistency than the surrounding pulmonary tissue. Smears from the lung nodule and axillary gland showed a number of very faintly stained yeast-like bodies about the size of the coccoid form of *B. pestis* along with numerous postmortem invaders. Smears from the inguinal abscess showed no bacteria, excepting some large postmortem invaders.

*Animal inoculations.*—A white rat, inoculated cutaneously with juice from the apparently consolidated portion of the squirrel's lung, was chloroformed in a dying condition on the 5th day. There was slight subcutaneous injection; no local reaction no buboes. The spleen was congested and soft. Pure cultures resembling *B. pestis* and which gave typical large clubbed involution forms on 3 per cent salt agar were obtained from the heart's blood and a similar culture was obtained from the spleen, though this was mixed with micrococci.

A guinea-pig inoculated cutaneously with lung tissue died in eight days with marked subcutaneous injection; large hemorrhagic and necrotic inguinal buboes; numerous minute areas of pneumonia in its lungs and foci of necrosis in the liver and spleen; the spleen was soft and congested. A pure culture resembling *B. pestis* was obtained from its spleen. This culture produced typical stalactites in oiled broth. A guinea-pig inoculated cutaneously from the squirrel's axillary gland died in eight days with the same anatomical findings described in the above pig. A culture from its spleen was sent to Passed Assistant Surgeon Geo. W. McCoy for confirmation.

*Squirrel No. 3.*—Shot on the Southern Pacific railroad tracks near the Morton Foundry, Contra Costa County, on August 25, 1908. Received for examination August 26.

The body was in fresh condition. There was an encapsulated abscess about  $10 \times 5 \times 5$  mm. in the left groin; the lungs showed hemorrhagic areas due to shot and appeared normal, excepting a yellowish white consolidated area about  $4 \times 4$  mm., circular and extending about 4 mm. into the substance of the middle portion of the ventral surface of the left anterior lobe.



The inguinal bubo contained a creamy pus which showed a few bipolar-staining rods about the size of *B. pestis* along with other larger rods. Smears from the pneumonic area in the lung showed a few bipolar-staining rods and coccoid forms resembling *B. pestis* along with numerous postmortem invaders.

*Animal inoculations.*—A white rat inoculated cutaneously with lung tissue died in three days. It showed subcutaneous injection and congestion of the internal organs. The spleen was dark and congested. Smears from the spleen and liver showed numerous bipolar-staining bacilli resembling *B. pestis*. Numerous colonies resembling those of *B. pestis* were obtained from the spleen and heart's blood on +1 agar slants and transplants gave typical involution forms on 3 per cent salt agar in 24 hours at 37° C. The culture from its heart's blood produced typical stalactites in oiled broth.

A guinea-pig inoculated cutaneously with lung tissue died in seven days, with the typical lesions of plague. Pure cultures resembling plague were obtained from its spleen and heart's blood.

*Squirrel No. 8.*—Found dead in a field near Bay Point, Contra Costa County, by Passed Assistant Surgeon W. C. Rucker on September 19, 1908. Received for examination September 20.

There was some postmortem decomposition; no subcutaneous injection; no enlargement or congestion of the cervical, axillary, inguinal, abdominal, or pelvic glands; thoracic cavity almost full of a clear serous exudate; lungs appeared normal, excepting two petechiae about 1 mm. in diameter on the dorsal surface of the left anterior lobe; the spleen was firm and slightly enlarged, of a very dark reddish, blue-black color and of irregular contour, owing to the presence of numerous irregular nodules. These irregularly rounded nodular areas were 2-4 mm. in diameter and of a yellowish-gray color with hyperemic borders (see Plate 4, Fig. 2). They were very numerous at both ends of the spleen where they became confluent. On section, these nodules were seen to extend deeply into the substance of the organ and were composed of a firm yellowish-white tissue. The liver was of a chrome-yellow color, due to phosphorus or arsenic (?); its capsule was smooth and of an oily luster. Scattered over its surface, about 25 irregularly rounded reddish areas 2-3 mm. in diameter were seen. These reddish areas were especially congested at their peripheries. Besides these, there was an equal number of yellowish areas 2-3 mm. in diameter which in some cases projected slightly from, in others receded from, the capsular surface. On opening one of those yellowish areas it was seen to contain a yellowish purulent

sticky material. There were four similar yellowish areas apparently embedded in the muscular substance of the diaphragm.

*Microscopic examinations.*—In smear preparations from the spleen nodules there was quite a number of coccoid bodies and fewer bipolar-staining rods resembling *B. pestis*. There was also quite a number of large rod-shaped post-mortem invaders.

In preparations of the pus from a small abscess near the costal border of the liver a few bipolar-staining rods resembling *B. pestis* were seen. No bacteria were found in lung smears.

*Animal inoculations.*—Guinea-pig No. 1. was inoculated cutaneously with tissue from a spleen nodule; appeared very ill for five days, preceding death on the eighth day; some subcutaneous injection; inguinal glands large and rather pale on section; spleen greatly enlarged, about six times normal size, and full of minute whitish foci of necrosis; liver swollen and full of similar minute granular foci of necrosis; lungs showed numerous grayish tubercular nodules, 1-3 mm. in diameter, scattered throughout the substance and beneath the plurae of all lobes. All these nodules were surrounded by hemorrhagic zones, like the nodules in the squirrel's spleen. Rods resembling *B. pestis* and coccoid forms were very numerous in its spleen and lungs but very few were found in the inguinal buboes.

Guinea-pig No. 2, was inoculated cutaneously with pus from a small abscess in the liver. It ate its food regularly though it appeared ill for about eight days preceding its death on the 15th day. It showed very slight subcutaneous injection and large inguinal buboes 15×10×10 mm. surrounded by a slightly congested zone and some periglandular serogelatinous exudate. On section the buboes appeared caseous. The spleen was of about normal size and contained 20 or 30 irregularly rounded whitish nodules resembling those in the squirrel's spleen, though smaller; the liver showed nothing noteworthy; there was a clear serous hydrothorax; the lungs were intensely congested and at the apex of the left anterior lobe was a yellowish-white area of consolidation; another irregularly rounded area of consolidation 10×6 mm. occurred in the dorsal center of the right middle lobe and still another 12×10 mm. near the posterior margin of the right posterior lobe. All these pneumonic areas were surrounded by zones of intensely congested pulmonary tissue resembling the areas described in the lungs of squirrel No. 1. Microscopically bacilli resembling *B. pestis* were found in greatest numbers in preparations from the pneumonic areas in the lungs. Pure cultures resembling *B. pestis* were obtained from the lungs, heart's blood, and spleen.

White rat No. 1 was inoculated cutaneously from the spleen of guinea-pig No. 1. It died of acute plague in three days, showing marked subcutaneous injection, and congestion of the internal organs. There were no pulmonary lesions. Bipolar-staining bacilli resembling *B. pestis* were very numerous in the spleen and heart's blood. Cultures resembled those of *B. pestis*.

Guinea-pig No. 3 was inoculated cutaneously from the spleen of guinea-pig No. 1, in order to see whether the character of the lesions would persist; it died on the tenth day, showing slight local reaction; large pale inguinal buboes. On section the buboes showed caseous centers with a narrow zone of congestion about the caseous material. The lungs were very intensely congested and all the lobes contained many irregularly rounded, yellowish-white nodules 2-4 mm. in diameter, which were firm on section. There were also numerous similar nodules  $\frac{1}{2}$ -1 mm. in the liver and the spleen was literally full of yellowish-white irregular nodules, 2-5 mm. in diameter, resembling those

in the spleen of squirrel No. 8. Bipolar-staining rods resembling *B. pestis* were scarce in the inguinal buboes but numerous in the lung nodules.

Guinea-pig No. 4 was inoculated cutaneously from the spleen of white rat No. 1 in order to see whether the virulence for guinea-pigs was increased. It died in six days showing marked subcutaneous injection; large caseous inguinal buboes surrounded by a hemorrhagic and serogelatinous exudate; a spleen about twice the normal size, soft and full of yellowish foci of necrosis; lungs not consolidated. Rods resembling *B. pestis* numerous in spleen and inguinal buboes.

White rat No. 2 was inoculated cutaneously with a piece of consolidated lung tissue from guinea-pig No. 2. It died in two and a half days, showing marked subcutaneous injection; congestion of the internal organs; hydrothorax; but no pneumonia. Rods resembling *B. pestis* were very numerous in the spleen and inguinal glands.

Guinea-pig No. 5 was inoculated cutaneously with consolidated lung tissue from guinea-pig No. 2 to see if the tendency to localize in the lungs would persist. It died in five days and showed large hemorrhagic inguinal buboes with caseous centers. Its spleen was about four times the normal size, soft and full of yellowish-white foci of necrosis. The liver was congested and showed numerous foci of necrosis. Its lungs were greatly congested and showed areas of hemorrhagic consolidation and many yellowish-white consolidated areas 1-3 mm. in diameter and many subpleural petechiae. Bipolar-staining bacilli resembling *B. pestis* were very numerous in the lungs, spleen, and buboes.

*Cultural studies.*—Cultures on +1 agar slants from the spleen nodules of squirrel No. 8 yielded numerous delicate, viscous colonies which when transplanted to artificial media showed the same cultural characters described for *B. pestis* under squirrel No. 1. Besides the fine colonies there grew a number of large white colonies of an actively motile bacillus which belonged to the colon group—coagulating milk and fermenting dextrose, levulose, lactose, saccharose, maltose, galactose, and mannite with gas-production. Cultures from the heart's blood of squirrel No. 8 yielded a few scattered delicate plague-like colonies.

#### REMARKS ON CERTAIN FEATURES OF NATURAL SQUIRREL PLAGUE. THE EXPERIMENTAL WORK OF DR. CURRIE.

It should be noted that in three of the four naturally infected squirrels, the lungs showed areas of consolidation due to *B. pestis*. In the case of squirrel No. 1, which showed the most marked pneumonia, the mucus of the posterior nares was proven to be infective. Then it will be noted that in the series of animal inoculations, under squirrel No. 8, the tendency for *B. pestis* to localize in the lungs was shown by a series of cutaneous passages through guinea-pigs. However, when this strain was passed through a white rat, which died of acute plague, and then back into a guinea-pig, this guinea-pig died with the anatomical and microscopical findings of acute plague. I do not know whether such a tendency to localize in the lungs has been observed in natural rat plague. However, it is stated that "Martini

was able to produce plague pneumonia in rats in 32 out of 36 inhalation experiments and thought that the virulence was exalted by passage from lung to lung; and when such cultures were injected intraperitoneally or subcutaneously they showed a great tendency to localize in the lungs."

It seems quite possible that human pneumonic plague may be due to infection with a strain of *B. pestis* having a tendency to localize in the lungs. On the other hand this tendency may be the expression of a greater resistance, for Klein<sup>1</sup> found that a certain number of white rats, guinea-pigs, and monkeys, insufficiently protected by Haffkine's prophylactic, developed plague pneumonia. He suggests that the first human case may arise in this way.

This peculiar tendency to localize in certain sites is well illustrated by a human case, H. 171, which was autopsied last winter in San Francisco. The only lesions at autopsy were a number of tumor-like nodules in the liver containing bipolar-staining bacilli. A culture from one of these nodules was kept by me and later turned over to Passed Assistant Surgeon George W. McCoy who proved it to be a virulent strain of plague.

My assistant, Mr. A. Venzke, who was with Passed Assistant Surgeon D. H. Currie in 1904, called my attention to the fact that Dr. Currie often noted pneumonia in his experimentally infected squirrels. These squirrels were identical with *O. beecheyi* according to Venzke. Through the kindness of Dr. Blue I am able to refer to Dr. Currie's experiments, which have never been published.

Out of nine experiments, we can draw the following data: When ground squirrels are inoculated subcutaneously or cutaneously with plague cultures or the organs of guinea-pigs or squirrels dead of plague, they die in from 44 hours to 10 days. Leaving out the squirrel which lasted for 10 days, the average time till death is 3.75 days. Among these inoculated squirrels, No. 4 died of primary plague pneumonia without noticeable septicemia; and No. 8, which lasted 10 days, died of bubonic, pneumonic, and septicemic plague.

Further, Dr. Currie experimented on transmission by "contact." Out of six squirrels associated with plague-sick squirrels, three died of plague, one of primary plague pneumonia, and the other two of

<sup>1</sup> *Bacteriology and Etiology of Oriental Plague*, 1906, p. 108.

pneumonic and septicemic plague. Another of the contacts was chloroformed and showed a plague bubo in the axilla.

In a single feeding experiment in which two squirrels were fed on carrot upon which a few drops of blood containing plague bacilli had been smeared, one died in four days with a bubo just anterior to and under the angle of the lower jaw with secondary plague septicemia.

I will quote one of the experiments as reported by Currie:

*Squirrels Nos. 14, 15, and 16.*—October 22, inoculated No. 14 by dermic method (that is, by rubbing the infectious material on the shaven but unbroken skin of the animal) in the skin of the back, using the spleen of a guinea-pig which had died from inoculation with No. 9. Placed No. 14 in cage with two healthy squirrels, Nos. 15 and 16. October 25 No. 14 died. Necropsy showed an extreme degree of plague dermatitis at site of inoculation, which had gone on to gangrene. The tissue showed necrosis and sloughing which in some places had exposed the spinal column. The neighboring glands were enlarged and inflamed and there was a plague septicemia. October 31 contact squirrel No. 15 died. Necropsy showed a plague dermatitis about the nostrils and double pneumonia due to the plague bacillus and a moderate plague septicemia. No glandular enlargements were present. November 7 contact squirrel No. 16 died (13 days after last contact with No. 14 and eight days after last contact with No. 15). Necropsy showed primary plague pneumonia. There were a few bacilli in the spleen and heart's blood.

#### THE RELATION OF PLAGUE IN SQUIRRELS TO THAT IN RATS AND MEN.

Apart from the danger of the gradual spread of squirrel plague across country through these all too numerous rodents and apart from the occasional transfer of plague from squirrel to man, it seems altogether likely that ground squirrels may act as a host for the *Bacillus pestis* in the interim between the more noticeable outbreaks in rats and men. Plague kept alive in ground squirrels might then be reintroduced into more thickly populated sections either by human or rat carriers. For example, a human having acquired infection during squirrel-hunting might reintroduce the infection among rats either in the form of plague-infested squirrel fleas or by himself, being then the source of infestation for human fleas. *P. irritans*, the human flea, has been found, sometimes in considerable numbers, on rats (*M. norvegicus*) on both sides of the bay.<sup>1</sup> As will be shown

<sup>1</sup> By M. B. Mitzmain, *Monthly Bull. California State Board of Health*, 1907, 3, p. 38; by R. W. Doane *loc. cit.*, *infra*, and by myself in Oakland.

The following will illustrate very well how a flea population may once again come into its own in a locality where active sanitary measures are frowned upon. Recently a rat with acute septicemic plague was caught in the basement of a vacant dwelling-house right in the center of Oakland. The house faced the street; it had a vacant building on one side, a Japanese market on the other, and these were surrounded

below we have absolute evidence that squirrel fleas occur on rats and that squirrel fleas occur on and are known to bite humans. A case which is quite apropos, even though the evidence that the man acquired his infection from squirrels is not complete, might be cited here.

Mr. S., a resident of Oakland, who worked in the sewers, died July 21, 1908. He was attended by two prominent physicians who pronounced his disease "typhoid pneumonia." As his death occurred in four days after the onset, Passed Assistant Surgeon J. D. Long, then in charge of the work in Oakland, demanded an autopsy, which he performed himself at night in spite of the threats of a mob of the man's relatives and friends. The case proved to be one of pneumonic and septicemic plague and was proven such by bacteriologic examination and guinea-pig inoculation by myself and independently by Passed Assistant Surgeon McCoy of San Francisco and Drs. P. S. Newsbaumer and R. A. Archibald of Oakland, Cal. No plague rats had been found in Oakland since the middle of April. Still, as only 9,361 rats had been examined since the middle of April, it seemed possible that the man might have acquired the infection in the sewers. However, the Oakland council refused to appropriate money for further plague work on the basis of this case, though they had said, "Wait until we have human cases!" Some of the members of the council said that this was not a fair case, probably manufactured to influence them, and then, anyway, they had absolute evidence that the man had been hunting squirrels in Contra Costa County shortly before his illness. Taking it for granted that the man acquired his infection in Contra Costa, it was no fault of the town council that subsequent infection of rats did not occur. Owing to the great sanitary clean-up, human fleas were scarcer in the bay region than they had ever been as far back as native sons could recollect.

by perfectly filthy shacks occupied by Chinese. The basement was riddled by rat runways and rat droppings could be detected on the first and second floors. It was so heavily infested with fleas that the dust upon the floors could be seen to pulsate with their movements. Four sheets of fly paper were placed on the floor of the basement for one minute and then removed. One of these sheets was speckled with 100 fleas. Another sheet caught 115 fleas; a third about 95 fleas, and the fourth about 75 fleas. The legs of the man entering the house were covered with fleas and he was able to bottle 67 in a short time. The majority of these fleas were *P. irritans* but in addition there were five which, according to Passed Assistant Surgeon Carrol Fox, were probably introduced from neighboring chicken yards. This species has been taken from fowls, sparrow nests, and occasionally from rats and men.

As the house had a rat population only, these fleas must have derived their nourishment from rats, and at least one of these was plague infected. It would be simply marvelous if something did not happen with the elements in such favorable conjunction.

In August, 1908, R. W. Doane<sup>1</sup> of Stanford University, published a note on certain collections of fleas sent to him. Out of 174 fleas, collected by Dr. Snow from rats caught on the campus of the university there were eight ground-squirrel fleas. "The eight specimens, which seem to be identical with Baker's *Hoplopsyllus anomalus*, which was originally described from a spermophile in southern Colorado, are interesting in that they seem to show a possible connection between rats and squirrels. Dr. Blue has often stated that should the plague ever become endemic here, it would probably spread from the rats to the ground squirrels, thus making it much more difficult to stamp out."

That there is more than a possible connection between rats and ground squirrels is well known. Ranchers have often observed that, especially in harvest time, Norway rats emigrate into the fields and then may be seen running in and out of ground squirrel holes. During the past few weeks of September and October the men trapping ground squirrels for me in the Berkeley Hills succeeded in trapping more Norway rats (*Mus norvegicus*) than any other species of rodents. The game traps were set well within the burrows of *Otospermophilus beecheyi*. The occurrence of *Hoplopsyllus anomalus* on *Otospermophilus beecheyi* has already been pointed out by Passed Assistant Surgeon Carroll Fox.<sup>2</sup> And in September, 1908, Passed Assistant Surgeon Geo. W. McCoy proved experimentally that the *H. anomalus* and *Ceratophyllus acutus* removed directly from a squirrel would bite a human being and suck his blood, thus confirming the reports of ground-squirrel hunters that they are often severely bitten by squirrel fleas.

This report should impress others with the fact that while the meager corps of sanitary officers with which this government is supplied have been and are still wide awake to the gravity and necessities of the plague situation, much remains to be done. There is no question in my mind nor in that of many others that, in spite of the apparent disappearance of plague, it still lurks hidden in some lair, perhaps and probably in the subterranean burrows of the ground squirrel and only the right combination of circumstances is required for its reap-

<sup>1</sup> *The Canadian Entomologist*, August, 1908, p. 303.

<sup>2</sup> *Public Health Reports*, September 25, 1908, p. 1371.

pearance in epidemic form. Why not kill off the ground squirrels? It would require a well-trained army to do that. The only really efficient method of attacking them at present is with the so-called "gopher-smokers," by means of which they may be driven from their holes with carbon bisulphide or sulphur dioxide. This method is almost worthless in the dry season when the surface crust is rent by deep cracks; and in the wet season one is soon anchored to the earth by adobe mud. Dr. Currie found them immune to Danysz virus, Rattin, and Squirrelin. I have experimented with a bacillus which produces a pseudotuberculous disease in guinea-pigs.<sup>1</sup> Dr. Theobald Smith<sup>2</sup> once suggested the possible usefulness of this germ in the extermination of spermophiles. However, the results of my experiments up to date are not at all encouraging. Judging from observations made at autopsies on ground squirrels and from data collected from ranchers few squirrels will take a lethal dose of arsenic or phosphorus. However, experimental work along this line may bring forth fruit.

Dr. Blue has recommended to the State Board of Health that "one of the best methods of ridding an area infested with ground squirrels is to encourage their natural enemies, such as the weasel, skunk, fox, and hawk. It has been observed by the Biological Survey that districts infested by these predatory animals have only a few ground squirrels. That in places where they have been killed off the squirrels abound in great numbers."

Of course, if there were no rats, plague among squirrels would be of comparative insignificance owing to their less intimate association with humans. The formation of societies for the extermination of the rat in various parts of the world shows that the race is cognizant of the dangers from that source. It is universally conceded that all the methods of poisoning and trapping yield only a temporary reduction in the number of rats in any community; and the best known bacterial viruses fail to kill more than half the rats feeding upon them, and leave behind a large number of naturally or artificially immunized rats. Officers of the Indian Medical Service have made use of the

<sup>1</sup> *Infra*, p. 519.

<sup>2</sup> *Jour. Med. Research*, 1903, 9, p. 286.



cat, the natural enemy of the rat, in certain villages in the Punjab, India, with some measure of success. And more recently Dr. Robert Koch has expressed his opinion to the effect that the only solution lies in the breeding and maintenance of a race of cats which are born ratters.

It seems to me, as it does to many others, that the only way in which humans can hope to bring about a permanent reduction in the rat population of the world must be brought about by entirely different means. In this country at least state and local governments must pass and enforce alterations in our building laws so that structures already standing may be altered, and future buildings so constructed that rats may not gain access to foodstuffs or breeding-places within them, and so also not come into intimate association with humans inhabiting them. Work of this character has already been begun in several parts of the world, but heretofore has been purely spasmodic and of a local nature.

#### SUMMARY AND CONCLUSION.

The *Arctomyiinae* and *Sciurinae* are numerous, diversified, and widely distributed in North America. Plague among these animals is known elsewhere in the world. Following the epidemiological and experimental observations of officers of the United States Public Health and Marine Hospital Service the actual demonstration of plague among the California ground squirrels has been made. The relation between squirrel plague and the origin of certain human cases has been demonstrated. The pathologic and bacteriologic features of squirrel plague are discussed. The probable origin in squirrel plague of the successive outbreaks on the Pacific Coast is indicated. The intercommunication between ground squirrels, rats, humans, and their fleas is referred to, along with the difficulties and gravity of the problems which now face the Public Health Service of the United States.

In conclusion I wish to express my indebtedness to Passed Assistant Surgeon Rupert Blue, commanding, to Passed Assistant Surgeon J. D. Long, formerly in charge of Alameda and Contra Costa counties, and to Passed Assistant Surgeons Geo. W. McCoy and Carrol Fox

of the plague laboratory in San Francisco, for without their generous assistance and collaboration it would have been impossible to have presented much of the information embodied in this article.

DESCRIPTION OF PLATE 4.

PLATE.—Fig. 1, right lung and heart of squirrel No. 1; Fig. 2, three-fourths of spleen of squirrel No. 8. For descriptions see text.

PLATE 4.



FIG. 1.

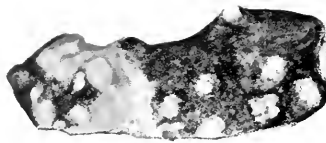
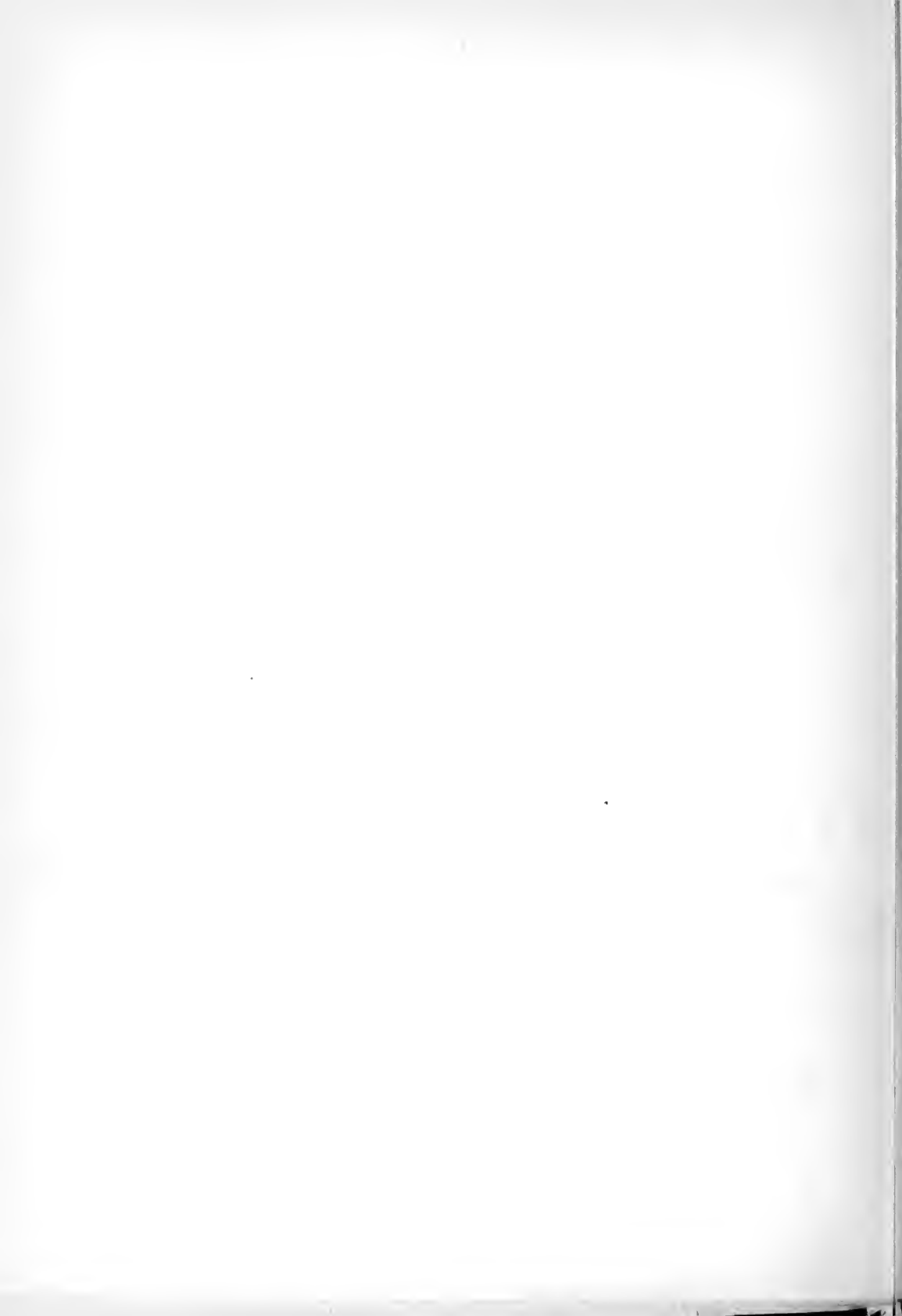


FIG. 2.



## FURTHER NOTES ON RAT LEPROSY AND ON THE FATE OF HUMAN AND RAT LEPROSY BACILLI IN FLIES.\*

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THE idea that parasitic insects might play some rôle in the transmission of leprosy seems to have existed for some time. More or less presumptive evidence in the case has accumulated, and this has been well reviewed by Nuttal:<sup>1</sup>

It appears that Linnaeus and Rolander considered that *Chlorops (Musca) leprae* was able to cause leprosy by its bite,<sup>2</sup> and Corredor<sup>3</sup> tells of flies in connection with leprosy, citing the case of an Indian who had lived some time with lepers and acquired leprosy, as he himself claimed, through the agency of flies. The insects gathered frequently in great numbers on the ulcers of his leprous comrades and some of these had bitten him. The first leprous ulcers appeared on the places where the insects had inflicted wounds.

Joly<sup>4</sup> says his teacher Sabrazes has long held the view that leprosy might be produced by a large number of small inoculations such as insects, especially parasites, may inflict. This, he considers, seems probable because of the large numbers of lepra bacilli which are present in the skin and ulcers in cases of cutaneous leprosy. Insects could scarcely avoid taking up the bacilli in sucking the blood or the exudations from the ulcers of leprous subjects and might transfer and inoculate the germs into healthy individuals. He seems inclined to attribute a part of the leprosy which prevails, especially among the poor and unclean classes, to the agency of cutaneous parasites which are often found amongst them. An observation of Boeck's of the presence of *Sarcoptes scabiei* in a case of cutaneous leprosy led Joly to conclude that these parasites might at times serve as carriers of the infection. It appears that these parasites are very frequently found in Norway in places where much leprosy exists; they, as also *Pediculi*, are usually present amongst the poorer classes in Algeria, which furnish the greater number of lepers. In Soudan, the *Sarcoptes* occur on almost all the dogs and often attack the natives, among whom there are numerous lepers. It seems to me that the possibility of this mode of transfer cannot be denied, but it is also conceivable that the pathological changes produced in the skin by the parasites may even favor the multiplication of the lepra bacilli. Finally, Sommers<sup>5</sup> of Buenos Ayres expresses

\* Received for publication Oct. 21, 1908.

<sup>1</sup> *Johns Hopkins Hosp. Rep.*, 1900, 8, p. 1.

<sup>2</sup> Blanchard, *Zoöl. Med.*, 2, p. 497.

<sup>3</sup> *Revista Med. de Bogotá*, reviewed by Polakowsky in *Deutsche med. Wchnschr.*, September 30, 1897 p. 646.

<sup>4</sup> *Thèse*, Bordeaux, 1898, p. 67.

<sup>5</sup> "Leprosy in the Argentine Republic," *Semana Medica*, June 23, 1898, reviewed in *Jour. Amer. Med. Assoc.*, Sept. 10, 1898, p. 618.

the belief that mosquitoes probably act as active agents in the spread of leprosy in warm countries, but that this is very unlikely.

Again, according to Baker<sup>1</sup> "the interest in this subject is further accentuated by the statement that Dr. Carrasquillo, of Bogota, has found the bacillus of Hansen in the intestinal contents of fleas. It is thus open to question if the fleas are not the agents for the communication of leprosy."

Further, Dr. W. J. Goodhue and his assistant, Father Joseph, working at the leper settlement at Molokai, H. T., made many dissections and stained preparations from mosquitoes and finally found lepra bacilli in the intestinal contents of a female *Culex pungens*. Later they found similar organisms in the common bedbug, *Cimex lectularius*.<sup>2</sup>

It is related that the British Leprosy Commission investigated the possible rôle played by insects with entirely negative results. I have been unable to consult their reports.

My interest was aroused in the possibility of blood-sucking insects playing some rôle in the transmission of leprosy during my studies upon the leprosy of rats. That this disease occurs among rats (*M. norvegicus*) on the Pacific Coast has already been pointed out by me.<sup>3</sup>

While detailed to plague work in Oakland, Cal., careful search was made for leprosy in rats in order to gain some idea as to its prevalence. During the four months between April 15 and July 15, 1908, 9,361 rats were dissected. Of these 20 (.00021 per cent) were infected with leprosy, nine of whom were females and six males; in five the sex was not recorded. The experience gained in recognizing the anatomical features of the disease resulted in the recognition of some cases of very recent infection. It seems probable therefore that few, if any, leper rats were overlooked in this series. Most of these rats, however, were very large, old rats, which showed such marked alopecia and superficial ulceration, with occasional atrophy of the tail or toes of the feet, as to excite one's suspicion at once. To illustrate the sickly and debilitated condition of rats in an advanced

<sup>1</sup> *Proceedings of the United States National Museum*, 1905, 29, p. 122.

<sup>2</sup> Referred to by Dr. E. S. Goodhue, *Amer. Med.*, 1907, 2, p. 593.

<sup>3</sup> *Jour. Amer. Med. Assoc.*, 1908, 50, p. 1903.

stage of this disease, I might relate that leper rat No. 13 was found staggering about in an apparently half-blind and defenseless condition on one of the public streets of Berkeley, in broad daylight. Judging from the results of experimental inoculation, the two early cases of naturally acquired leprosy examined by me must have been of six to eight months' duration. The only lesions present were in the axillary glands in one case and in the inguinal in the other, and in the subcutis immediately adjacent to these regions. The submaxillary and cervical glands have been never found infected, even in the most advanced cases. Various possible modes by which the disease may be transmitted from rat to rat suggest themselves. Myriads of lepra bacilli lie just beneath the Malpighian layer of the epidermis, in advanced cases. These might be transferred by blood-sucking parasites, *Siphonaptera*, *Pediculac*, or *Tabanidae*, which so frequently attack sickly animals in unusual numbers. Then, as in human leprosy, the bacilli sometimes escape in considerable numbers with the nasal secretion. In my series, out of six examinations of the mucosa of the posterior nares lepra bacilli were found three times; twice they were numerous and once few in number.

Again, a leper rat's teeth might become instruments of inoculation, either through contamination from the posterior nares or during the rat's process of ridding itself of insect parasites, or of licking its ulcers. Further, infection may occur through the gastro-intestinal tract, either through feeding upon the bodies of dead leper rats or through food-stuffs contaminated by leper rats. McCoy<sup>1</sup> reports finding lepra bacilli in the bladder of one rat. Examination of the contents and mucosa of the bladder in four advanced cases, made by myself, gave negative results.

The character of the early lesions present in naturally acquired cases of this disease seems to point to inoculation through the skin. Ectoparasites were very scarce upon the rats as they were all brought in dead. One *Ceratophyllus fasciatus*, one *Ctenopsyllus musculi*, one rat louse (*Haematopinus acanthopus*) and one cone-nosed arachnid were carefully ground up on slides and stained for lepra bacilli without results. Only one nematode has been encountered in sites where the lepra bacilli occur and this is a filaria-like worm, about

<sup>1</sup> *Public Health Reports*, July, 1908, 23.

20 mm. long, sometimes found in the posterior nares of the *M. norvegicus*.

The presence of enormous numbers of lepra bacilli in the excretions of the cutaneous ulcers suggested the possibility of their being taken up by flies. The ability of lepra bacilli to multiply and persist within a fly after the manner of tubercle bacilli<sup>1</sup> could only be affirmed or doubted after actual observations on this point.

#### EXPERIMENTS WITH FLIES.

1. *The fate of lepra bacilli in flies fed on the carcass of a leper rat.*—The carcass of leper rat No. 4 was found on May 20, 1908, in a cellar beneath a grocery store. It was apparently two or three days old and badly fly-blown—a large female *M. norvegicus*, showing marked alopecia; multiple ulcerations of the skin, which was thickened especially about the regions of the axillae, and hyperplasia of the cutaneous, axillary, and inguinal glands were present. Smears showed enormous numbers of lepra bacilli in the subcutis, cutaneous ulcers, and axillary and inguinal glands. After dissection the carcass was placed in a large glass jar, and exposed to flies. When a number of flies had collected within the jar, it was quickly screened. The flies were chiefly green bottle flies (*Lucilia Caesar*), blow flies (*Calliphora vomitoria*), and a few house flies (*Musca domestica*). After feeding upon the rat's carcass, the flies deposited specks upon the sides of the glass jar. Examination of the specks deposited during the first 24 hours after feeding showed that the flies took up enormous numbers of lepra bacilli and deposited them with their feces. These flies were then removed to a clean jar and fed upon the liver of a normal rat. Examination of the specks deposited during the second 24 hours showed that the lepra bacilli were almost completely voided during the first portion of this period, i. e., the old dried specks were the only ones which contained acid-proof bacilli and even these contained them in greatly diminished numbers. The fresh moist specks (deposited 40–48 hours after the ingestion of lepra bacilli) were almost invariably clear, though occasionally two or three acid-proof bacilli resembling lepra bacilli could be found.

<sup>1</sup> See "Flies and Tuberculosis," *Publication of the Mass. Gen. Hosp.*, 1906, 1, p. 118, for the work of F. T. Lord and his predecessors.



2. *The fate of leprosia bacilli in the larvae, pupae, and imagoes of flies hatched in the carcass of a leprosia rat.*—Larvae which had hatched out in the rat were washed by shaking in repeated changes of salt solution or broth and dissected. The vast majority of those examined contained enormous numbers of leprosia bacilli. To determine whether the bacilli would persist in the insects through the stage of pupation and appear in the imagoes, two series of experiments were performed:

*Series 1: To determine whether the bacilli would persist when the larvae were fed upon uninfected meat.*—One hundred fourteen large and small larvae were removed from the carcass of the leprosia rat, placed in a clean jar and from time to time fed on the livers of normal guinea-pigs and rats. Examinations of some of these larvae were made at intervals after their removal, e. g., two larvae examined five days and one on the sixth day after removal showed no leprosia bacilli. The larvae and pupae were kept at room temperature. The pupae were kept separated in flasks and when the flies hatched out they were fed on uninfected meat and their specks examined for leprosia bacilli. The following table gives some of the details of these observations:

TABLE I

No. Pupa	No. Days after Rat Was Fly-blown till Pupa Appeared	No. Days after Pupation until Fly Hatched Out	Kind of Fly	Age in Days of Fly When Specks Were Examined	Results
1.....	10	23	<i>C. vomitoria</i>	1	o Fly died.
2.....	10	26	"	5	o (Died.)
3.....	10	11	"	1	o (Died on 5th day.)
4.....	12	26	"	4	4 leprosia bacilli found in speck.
5.....	13	25	"	1	Shown quite a number of leprosia bacilli in clumps. The bacilli appeared quite segmented.
6-15.....	15	13-14	"	1	o

\* N. B.—This fly was fed on meat and its specks examined a few hours later showed no leprosia bacilli.

*Series 2: The fate of leprosia bacilli in the larvae fed almost continuously on the carcass of a leprosia rat and in the pupae and imagoes developing therefrom.*—The rat carcass alive with maggots was kept at outdoor temperature in a screened jar. A drop in the temperature delayed pupation. Three larvae were examined 12 days from the beginning of the experiment and all contained numerous leprosia bacilli. Eight pupae (four partially dried up and four normal) were examined 31 days from the beginning of the experiment and all contained numerous leprosia bacilli; in two they were very numerous.

Twenty-two blow flies (*C. vomitoria*) hatched out on the 43d day from the beginning of the experiment. The specks of 15 of these, deposited during the first 24 hours after hatching, contained no leprosia bacilli. The specks of six others, deposited during the first three days after hatching, contained no leprosia bacilli. One fly died without depositing any specks and this one with a number of others was dissected with negative results.

Thousands of pupae in the jar remained unhatched and were under observation for a month and a half. Some of these were dissected and found to contain numerous

lepra bacilli on the 43d day from the beginning of the experiment. So it would seem as if these heavily infested pupae were incapable of undergoing further development.

3. *The fate of lepra bacilli taken up by flies from the leprous ulcers on a human case.*—While visiting the lepers at the Alameda County Infirmary, on June 9, 1908, I caught two house flies (*Musca domestica*) upon the face of "Frank," an advanced case of nodular leprosy with ulceration of the nodules about the nose. The specks deposited in the vial within an hour after the flies were caught were examined and in one speck two lepra-like bacilli were found. During the first 24 hours after their capture the flies deposited numerous specks upon the sides of the vial. Five out of six of the specks examined showed considerable numbers of lepra-like bacilli, occurring singly and in clumps of 20-50 rods. The flies were transferred<sup>1</sup> to a clean flask and within an hour and a half there were three specks in the flask. These were deposited on the morning of June 11, or 1½ days from the time the flies were captured. Two of these specks were examined and both contained many lepra-like bacilli. The flies were then separated and it was found that only one of them, No. 2, was infested with acid-proof bacilli. The specks deposited by this fly on June 12, three days after its capture, also contained numerous lepra-like bacilli. Six specks deposited by this same fly between the third and sixth day of its captivity were carefully examined and only one lepra-like bacillus was found. The fly died on the sixth day.

4. *Determination of the number of lepra-like bacilli in a single fly speck.*—One of the specks deposited by fly No. 2 on the third day of its captivity was carefully removed and emulsified, on a slide, with a small drop of a suspension of typhoid bacilli. This was then spread over a square area with the point of a needle, stained by the tubercle method with a contrast of Löffler's methylene blue, and the number of acid-proof bacilli counted with the aid of a Zeiss mechanical stage. There were counted 1,115 lepra-like bacilli, but this does not account for the real total owing to the fact that the bacilli often occurred in clumps and it was impossible to count all of them.

5. *The determination of the probable nature of these acid-proof bacilli.*—The question of course is, May not these have been simply

<sup>1</sup> The transfer of flies from flask to flask is a matter of clocklike precision and simplicity if it be remembered that flies are strongly heliotropic.

tubercle bacilli or even perhaps some other acid-proof bacillus than the lepra bacillus? No attempt was made to cultivate these bacilli. However, numerous specks deposited during the first and second days of the fly's captivity were emulsified in salt solution. This suspension, which showed quite a large number of lepra-like bacilli, was injected, on June 11, into the abdominal subcutaneous tissue of a guinea-pig weighing 540 grms. On July 11 it weighed 570 grms. and showed no local reaction, nor enlargement of the glands. On August 8 it weighed 480 grms. and showed no signs of infection. On August 22 it weighed 502 grms. On September 14 it weighed 540 grms. On September 10 it was given 0.03 mg. of Koch's old tuberculin subcutaneously without a reaction. It was chloroformed and found perfectly normal.

#### THE RELATION OF RAT TO HUMAN LEPROSY.

There is no evidence that human leprosy and rat leprosy are identical or that human beings need fear infection from leper rats. A single experiment of mine may be detailed here though it proves nothing one way or the other to my mind:

Centrifuged and washed suspensions of rat lepra bacilli (leper rat No. 8) were mixed with 24-hour-old serum from three cases of human leprosy (one very advanced nodular case; one well-developed macular case, and one of anaesthetic type) in dilutions of 1:5, 1:10, 1:20, 1:40, 1:80, with controls of normal human serum. There was no agglutination in any case in one hour at room temperature and only in one case (at 1:5) when examined after 24 hours.

#### SUMMARY.

Most of the evidence bearing on the possible rôle of insects in the transmission of leprosy may be classified as purely *a priori* or as evidence based simply upon the finding of lepra-like bacilli in certain insects. It is evident that the simple taking-up of parasites by an insect does not necessarily imply that the insect plays a rôle in their transmission from one host to another. Actual experiments can only demonstrate this.

The leprosy disease of rats furnishes material for work of a comparative nature and these experiments deal wholly with the fate of

rat and human lepra-bacilli when ingested by flies or by the larvae of flies.

1. Flies (*C. vomitoria*, *L. Caesar*, *M. domestica*) take up enormous numbers of lepra bacilli from the carcass of a leper rat and deposit them with their feces; but the bacilli apparently do not multiply in the flies as the latter are clear of bacilli in less than 48 hours.

2. The larvae of *C. vomitoria*, hatched out in the carcass of a leper rat, become heavily infested with lepra bacilli.

a) If such larvae be removed and fed on uninfected meat they soon pass out most of the lepra bacilli. Such larvae pupate and the flies hatching therefrom are generally uninfected. Occasionally a fly may deposit a few lepra bacilli after emerging from its pupa case—but is apparently not infested in the real sense of the term.

b) If the larvae of *C. vomitoria* be fed almost continuously on the carcass of a leper rat they remain heavily infested with lepra bacilli. When they pupate, the heavily infested pupae seem to be incapable of undergoing further development.

3. A fly (*M. domestica*) caught on the face of a human leper was found to be infested with lepra-like bacilli. These were few in number at the beginning of the observation, but on the third day more than 1,115 lepra-like bacilli were present in each speck deposited. However, only one bacillus was found in the specks deposited between the third and sixth days. The acid-proof bacilli in this fly were not infective when injected into the subcutaneous tissue of a guinea-pig.

4. The agglutinating action of blood serum from three human lepers was tested on washed suspensions of rat-lepra bacilli with practically negative results.

## STREPTOCOCCI OCCURRING AS DIPLOCOCCI IN RATS.

(*M. norvegicus*.)\*

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THE most striking features of the following infection encountered in two rats are the presence in the affected tissues of numerous diplococci morphologically resembling the meningococcus; diplococemia, and the presence of a marked subcutaneous edema.

While the micrococci occur mainly in pairs, occasionally short chains may be detected in smears from the tissues. They take the ordinary aniline dyes readily, and retain the stain in Gram's method. They grow readily upon transfer from the tissues to +1 agar slants.

Their biochemical characters, as far as determined, may be summed up briefly as follows: Upon +1 agar slants, in 24 hours, at 37°, pin-point to 0.25 mm. circular, white colonies with thin even edges appear. In 48 hours the colonies are larger and finely granular under low magnification, with finely notched or smooth edges. Colonies upon +1 rabbit's blood-agar plates are surrounded by a zone of hemolysis; the growth is more delicate upon rabbit's blood-agar slants than upon plain agar. Cultures may be kept viable for months on rabbit's blood agar, but not so on plain agar. Slight flocculent growth in +1 broth. Delicate white growth, without liquefaction, in +1.5 gelatin (18-25°) in the case of culture 2,854; no growth in the case of culture 22,398. No visible change in +1 milk during seven days' growth. Acid is produced in +1 litmus broth, containing 1 per cent dextrose, levulose, saccharose, maltose, and galactose; mannite and inulin are not fermented. Culture 2,854 produces acid from lactose while 22,398 does not. As usual the growth is very much more luxuriant and longer chains are formed in the media containing fermentable carbohydrates. Experimentally the micro-organisms were pathogenic to white rats but not to guinea-pigs nor rabbits.

Notes on the original infected rats read as follows:

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I. ADULT FEMALE *M. norvegicus* 22,398.

All the subcutaneous tissues are infiltrated with a marked serogelatinous edema, otherwise no noticeable abnormality is shown. Brain and serous membranes are normal. Smears from the heart's blood show enormous numbers of meningococcus-like diplococci, not seen in phagocytes; also trypanosomata which are alive in fresh preparations. Numerous isolated colonies of the cocci were obtained on +1 agar slants.

*Animal inoculations.*—A guinea-pig inoculated intraperitoneally with a physiological salt solution emulsion of the heart's blood remained well for a month. No trypanosomes appeared in the heart's blood. Chloroformed and found normal.

An adult white rat inoculated intraperitoneally with +1 broth emulsion of a 48-hour +1 agar slant culture, grown directly from the heart's blood of rat 22,398 was chloroformed six days later and found perfectly normal.

An adult white rat inoculated intraperitoneally with a +1 broth emulsion of a +1 rabbit's blood-agar slant culture, two removes from the heart's blood of rat 22,398, died in less than 24 hours. It showed subcutaneous injection, and a small amount of bloody fluid in peritoneal cavity; spleen, liver, kidneys, testicles, and lungs were intensely congested; fibrinous specks on pleura of lungs; serous exudation in thorax, brain congested, spinal cord normal. Microscopically diplococci were very numerous in the peritoneal exudate and occurred chiefly within phagocytes; here a few chains of two to four diplococci were seen. Numerous cocci in diplococcus form only were seen in preparations from the spleen and pleural exudate. None were seen in smears of the heart's blood. The micro-organism was recovered from the peritoneal exudate, spleen, and heart's blood.

An adult white rat inoculated subcutaneously with a +1 rabbit's blood-agar slant culture, two removes (48 hours) from the heart's blood of rat 22,398, appeared sick for a day after the injection but recovered and was chloroformed and found normal four days later.

A half-grown Belgian hare was inoculated intravenously with a broth emulsion of one rabbit's blood-agar slant culture, two removes (48 hours) on blood agar from heart's blood of rat 22,398. It

remained well and was chloroformed and found normal four days later.

The animals were chloroformed and examined in so comparatively short a time after inoculation owing to my transfer from San Francisco to Oakland.

## II. ADULT PREGNANT *M. norvegicus* 2,854.

This rat was caught and kept alive in a cage for four days when it died. It had a wound on the anterior aspect of left thigh. On dissection the subcutaneous tissues of the whole left half of the body were found infiltrated with a sero-gelatinous exudate and appeared somewhat hyperemic. The left inguinal glands were enlarged to 4-5 mm. in diameter, were firm and white on section, and imbedded in a sero-gelatinous periglandular exudate. The submaxillary and right and left cervical glands presented a similar appearance. The exudate also extended down the left thigh. The right side of the body was not so affected. The peritoneal cavity contained some fluid.

Smears from the affected glands showed enormous numbers of biscuit-shaped diplococci, rarely in leucocytes, and rarely in chains of four to six diplococci. No micro-organisms were found in preparations from the spleen and heart's blood.

Pure cultures of the cocci were obtained from the left inguinal gland on rabbit's blood-agar plates. Many colonies of similar cocci also grew on agar slants inoculated from the peritoneal fluid, spleen, and heart's blood.

*Animal inoculations.*—A broth emulsion of the left inguinal gland was used to inoculate two guinea-pigs, one subcutaneously, the other intraperitoneally; a white rat subcutaneously; a wild rat (*M. norvegicus*) subcutaneously; a Belgian hare intravenously. The guinea-pigs and hare were chloroformed 23 days later in a healthy condition and found normal. The wild rat died in 23 days from some unknown cause.

The white rat died in 15 days. There was an abscess at the point of inoculation; marked general subcutaneous congestion with sero-gelatinous exudation in both groins; abdomen distended with much bloody fibrino-purulent exudate and abdominal organs bound together

with the same. Cocci were very numerous in the peritoneal exudate and here the formation of chains was more marked than in the original rat. Probably the inoculation was partly intraperitoneally.

F. Kutschera<sup>1</sup> described cultures isolated from white mice which succumbed to a streptococcus epizootic. Owing to the differences in cultural procedures adopted by this worker and myself, one can only point to the following, differential characters between the two strains: In the epizootic described by Kutschera, the lesions were essentially of a suppurative character, due to a mixed staphylococcus and streptococcus infection; the streptococci were pathogenic for white mice, white rats, and rabbits, while guinea-pigs were refractive. The streptococci isolated from this form of infection in *M. norvegicus* occurred almost entirely as kidney-shaped diplococci in the tissues of the naturally infected animals; the infection was characterized by a marked subcutaneous edema and sero-gelatinous exudate; the cocci were non-pathogenic for rabbits as well as for guinea-pigs.

<sup>1</sup> *Centralbl. f. Bakt., Orig.*, 1928, 46, p. 671.



## EXPERIMENTS ON THE USE OF *BACILLUS PESTIS-CAVIAE* AS A RAT VIRUS.\*

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THE following details are drawn from notes kept on a series of experiments conducted at the Federal Laboratory between April 15 and November, 1908. The experiments with a commercial ratticide known as "Azoa" were undertaken at the request of Passed Assistant Surgeon J. D. Long, then in charge of the plague situation in Oakland, Cal., and at the request of the representatives of Parke, Davis & Co., who had asked for a fair experimental trial of their virus. This and subsequent experimental work was prosecuted with the aid of federal money, obtained by Passed Assistant Surgeon Rupert Blue, commanding.

### EXPERIMENTS WITH A COMMERCIAL VIRUS KNOWN AS AZOA.

*Series I.*—The following feeding experiment as detailed below was commenced before my arrival in Oakland. I was informed that the 12 rats (*M. norvegicus*) fed on azoa received the virus on the dates indicated below, each feeding for the 12 consisting of 4 oz. (124 gms.) of azoa, lot No. 010916-E, thoroughly mixed with 32 oz. (992 gms.) of dry rolled oats.

Four of the rats set aside for controls died before the feeding was commenced, probably from arsenical poisoning. All the rats were fed on rolled barley, daily, excepting Sundays when they were not fed at all, and excepting one feeding of raw meat (date not given) and excepting the dates when 12 of the rats were fed azoa.

Ten of the rats which died after feeding on azoa were examined 12-18 hours after death. Only four of these showed noticeable abnormality, some enlargement of the spleen and liver with numerous yellowish-white foci of necrosis scattered beneath the capsule of these organs. Microscopic examinations failed to reveal anything noteworthy excepting the rare occurrence of a bipolar staining diplobacillus in liver and spleen smears. Cultures from the spleens of these rats,

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TABLE 1.  
AZOA FED RATS.

No.	DATE CAUGHT	FEEDINGS			NUMBER OF DAYS AFTER LAST FEEDING TILL DEATH
		1st	2d	3d	
1.....	April 8	April 9	April 11	April 15	9
2.....	" 8	" 9	" 11	" 15	9
3.....	" 8	" 9	" 11	" 15	10
4.....	" 8	" 9	" 11	" 15	6
5.....	" 8	" 9	" 11	" 15	8
6.....	" 8	" 9	" 11	" 15	8
7.....	" 7	" 8	" 11	" 15	
8.....	" 7	" 8	" 11	" 15	8
9.....	" 7	" 8	" 11	" 15	6
10.....	" 7	" 8	" 11	" 15	8
11.....	" 7	" 8	" 11	" 15	5
12.....	" 8	" 9	" 11	" 15	6

CONTROL RATS.

No.	Date Caught	Date Died	Cause of Death	Still Alive
1.....	April 7	April 12	Probably arsenic	
2.....	" 7	" 12	" "	
3.....	" 8	" 12	" "	
4.....	" 8	" 12	" "	
5.....	" 7			April 27
6.....	" 7			" 27
7.....	" 8	" 14	Not determined	
8.....	" 8			" 27
9.....	" 8	" 15	" "	
10.....	" 7			" 27
11.....	" 8			" 27
12.....	" 7			" 27

however, uniformly yielded, in 24 hours, from 20 to 50 similar ring-like colonies. As will be seen below, they all died of infection with *B. pestis-caviar*. The six remaining controls were chloroformed at the close of the experiment and cultures from their spleens remained sterile.

In order to check the results already obtained and to prove whether the disease was transmissible by feeding rats on the organs of rats supposedly dead from azoa, another series was started.

*Series II.*—On May 30, 35 adult *M. norvegicus* were collected, and each rat was placed in a separate cage-trap supplied with firmly secured feed and water pans. They were numbered from 13 to and including 47. The traps were then arranged on a sawdust bed in a room sealed and locked with a yale lock. During the first four days, eight of the rats died, Nos. 13, 14, 15, 17, 18, 23, 28, and 31. Rat 18 had the leprosy-like disease and No. 31 died of an infection due to streptococci appearing as diplococci in the tissues. Rat 28 had a

yellow liver probably due to arsenic. The cause of death was not ascertained in the other instances. All of the remaining rats were fed on cracked barley one day and on cabbage leaves the next for a week, and were watered every day. As they now all appeared healthy and lively, they were separated into two lots of 12 each; 12 azoa rats, and 12 controls. They were all starved one day, and then the azoa rats received their first feeding of 4 oz. (124 gms.) of azoa, lot No. 010916-E, thoroughly mixed with 32 oz. (992 gms.) of dry rolled oats. The 12 controls were fed on 992 gms. of dry rolled oats.

The details of these feedings by dates are as follows:

- 1st feeding, June 8, Fed most of the azoa mixture to the azoa rats; watered.  
Fed most of the plain oatmeal to the control rats; watered.  
June 9, Fed rest of the azoa mixture to the azoa rats; watered.  
Fed rest of the plain oatmeal to the control rats; watered.  
June 10, Starved all the rats; watered.
- 2d feeding, June 11, and 12, Repeated feeding as per 1st feeding; watered.  
June 13, All rats fed on cabbage and watered.  
June 14, All rats starved.
- 3d feeding, June 15, and 16, Repeated feeding as per 1st feeding; watered.

The rats of this series were fed rolled oats one day and cabbage the next and watered daily. Excepting one, which had a bad case of scabies of the ears and neck, and rat 20 which died on the twentieth day after the last feeding, they all remained in perfect health. Rat 20 showed no anatomical changes but agar slants from its spleen showed numerous colonies which were proven to be those of *B. pestis-caviae*. They grew fat for over two months when they were chloroformed and their spleens proven sterile culturally.

Having in mind the work of Weiske on the oxalic acid poisoning of rabbits kept on a diet of oats alone, and the more recent work of Holst and Frolich<sup>1</sup> on the toxic action of a single diet on guinea-pigs, I started to determine whether experimental rats kept on a barley or oats diet alone might either die of a scurvy-like disease or become susceptible to invasion with a micro-organism, whereas when fed cabbage (which has been shown to neutralize the toxic action of oats, barley, etc.) they might survive. It was with this possibility in view that cabbage was introduced with the diet in Series II. However, experiments showed that rats, *M. norvegicus*, are not injured

<sup>1</sup> *Jour. Hyg.*, 1907, 5, p. 663.

in the least by being kept on a single diet of oats and barley. For example, six adult rats were fed daily on barley and water for two months. They grew fat and sleek and showed no abnormalities when chloroformed and carefully dissected.

In another series six rats were fed on azoa, according to the plan detailed under Series II, and then kept on a barley-and-water diet for 20 days, when they were chloroformed. They all appeared normal at necropsy and cultures from their spleens remained sterile.

Again four rats were kept on a diet of rolled oats and water for over a month with apparent benefit.

*B. pestis-caviae* AND ITS RELATION TO THE VIRUS OF AZOA.

During the course of these experiments, I encountered a pseudotuberculous disease in my stock of guinea-pigs. The only lesions of this disease I have encountered are the occurrence of a few or many firm, yellowish-white nodules in the spleen. These vary from less than 1 to 5 or 6 mm. in diameter. On section, they contain a semi-purulent or caseous material. Smears from these nodules show considerable numbers of rods with rounded ends. When fixed by heat and stained with carbol-thionin most of them show excellent bipolar staining. When measured (Zeiss  $\frac{1}{12}$  oc. microm. 6) they are most frequently  $1.8 \times 0.8 \mu$  though smaller coccoid forms  $0.8 \times 0.8 \mu$  and longer forms up to  $2.8 \times 0.8 \mu$  may be seen. They do not retain the stain in Gram's method.

As will be detailed below cultural studies, agglutination, and animal experiments show that the virus of azoa is identical with that of this pseudotuberculous disease of guinea-pigs. (It is highly improbable that these stock guinea-pigs obtained their infection from azoa, for they were kept in a separate building from the one in which the azoa experiments were conducted. The dealer from whom the guinea-pigs were obtained lived on the outskirts of the city and had raised them himself. He had never used a rat virus about his premises.)

This disease has been known since 1896. It was first described by Theobald Smith and J. R. Stewart.<sup>1</sup> In a later reference<sup>2</sup> it is described by Dr. Smith as "a bacillus isolated from a guinea-pig in 1896, and then called pseudotuberculosis, owing to the presence of

<sup>1</sup> *Jour. Boston Soc. Med. Sci.*, 1897, p. 12.

<sup>2</sup> Smith and Reagh, *Jour. Med. Res.*, 1903, 9, p. 273.

large tubercle-like foci in the liver and spleen which were necrotic-suppurative in character. This is evidently the same as the bacillus isolated by Dr. Edward P. Carter at Johns Hopkins University in 1897, a culture of which was kindly sent to me by Dr. Carter, and another recently by Dr. Harris, for comparison with our bacillus. As Dr. Carter did not publish his studies upon this organism, we may state here that in a personal communication Dr. Carter called it the bacillus of infectious endometritis in guinea-pigs. It was isolated from a spontaneous epizootic in which the females chiefly were carried off by it. They always showed an acute endometritis. A study of our bacillus and of Dr. Carter's bacillus showed no appreciable differences." Dr. Smith tells me that the Rockefeller Institute recently lost its entire stock of guinea-pigs from this disease.

So far as is known the original host of the germ seems to be the guinea-pig (*Cavia Aperca*). I have called it *Bacillus cholera-caviae* in a preliminary note<sup>1</sup> but on the recommendation of Dr. Smith it has been changed to *Bacillus pestis-caviae*, "since the disease may appear as multiple spleen and liver abscesses or as a puerperal disease."

#### BIOCHEMICAL CHARACTERS OF *B. pestis-caviae*.

*Cultural characters.*—When plated in agar<sup>2</sup> or litmus-lactose agar, circular, white, slightly elevated colonies, punctuate to 0.5 mm. to 1 mm., appear in 24 hours at 37° C. When well separated they may spread out to 3 or 4 mm. in diameter. They appear light brownish by transmitted light and produce no change in lactose. On stroke cultures made on agar slants from the tissues of infected animals the colonies are of the same character when well separated. If very numerous and closely aggregated they appear as minute transparent colonies. The growth is not viscous. Quite frequently the growth from animal tissues appears in the form of "ring-colonies" the bacteria being piled up in a white ring about a clear central space.<sup>3</sup> The organism produces alkali rapidly and this fact may be taken advan-

<sup>1</sup> *Public Health Reports*, November, 1908.

<sup>2</sup> Unless otherwise mentioned the reaction of the media was (+1) 1 per cent acid to phenolphthalein.

<sup>3</sup> This phenomenon has been noted before by Theobald Smith and Reed and Carroll (*Jour. Exper. Med.*, 1900, 5, p. 233) in the case of the colonies of *B. cholera-suum* and *B. icteroides* and I have noted it in the colonies of *B. coli* inoculated directly from the tissues of ground squirrels and humans.

tage of when it is mixed with acid producers on litmus-lactose-agar plates.

Sugar-free broth is evenly clouded and a faint but distinct indol reaction can be obtained after 10 days' growth at 37° C. In young cultures the bacilli are quite as actively motile as typhoid bacilli. In old broth cultures rather coarse stalactites may be produced.

There is good growth in +1.5 gelatin at 25-28° C. without any liquefaction during two months' observation. There is no visible change in milk cultures for several days but in 7 or 10 days at 37° C. the culture appears opalescent, and later it is turned to a clear, yellowish fluid.

Dextrose, levulose, maltose, mannite, and galactose are fermented with gas production ( $H/CO_2 = 2/1$ ). No acid nor gas is produced from lactose, saccharose, nor inulin. As in the case of many bacteria, growth is much more luxuriant when a fermentable carbohydrate is present.

In order to determine the systematic position of *B. pestis-caviae* and its relationship to the virus of azoö, a careful comparative study was made of the following cultures: from the spleens of rats 2, 5, 7, and 9, which died in the azoö feeding experiment (Series I); from the spleen of rat 12, which died from a subcutaneous inoculation with an emulsion of azoö; from the spleen of rat 20 (feeding experiment Series II), a culture isolated from azoö itself by plating in litmus lactose agar; from the pseudotuberculous spleen nodules of two guinea-pigs (July 8 and July 13); a culture of Danysz virus for rats, brought by me from Manila where I had myself transplanted it from the original tube from the Krål Laboratory; a strain of what appeared to be *B. enteritidis* isolated by myself from the liver of human case 14; and a strain of the hog-cholera bacillus obtained from the Hygienic Laboratory, Washington. All of these cultures correspond in every detail as above described for *B. pestis-caviae* excepting the lack of indol production in the case of *B. cholera-suum*.

Rabbits were immunized for a series of inter-agglutination relationship tests but I have had time only to complete one of the series; a Belgian hare was immunized with the culture from the spleen of rat 2 which died in the azoö feeding experiment (Series I). In all, 11 c.c. of broth cultures killed at 60° C. were injected during a

month and a half. The cultures to be tested were grown for 24 hours in sugar-free broth, brought up to as nearly a uniform density as could be determined with the naked eye, and 0.5 c.c. of each culture mixed with an equal amount of the diluted serum and kept at 37° C. for one hour. The following table gives the results of the first test with cultures; spleen azoia rat 2 (Series I); spleen azoia rat 20 (Series II); *B. pestis-caviae* (spleen guinea-pig of July 13); Danysz bacillus; and hog cholera.

TABLE 2.

CULTURES	DILUTIONS OF SERUM					
	$\frac{1}{10}$	$\frac{1}{20}$	$\frac{1}{40}$	$\frac{1}{80}$	$\frac{1}{160}$	$\frac{1}{320}$
2 .....	++	++	+	+	+	+
Controls .....	-	-	-	-	-	-
20 .....	++	++	+	+	+	+
Controls .....	-	-	-	-	-	-
Guinea-pig, July 13 .....	++	++	+	+	+	+
Controls .....	-	-	-	-	-	-
Danysz bacillus .....	+	+	±	-	-	-
Controls .....	-	-	-	-	-	-
Hog cholera .....	++	++	+	±	-	-
Controls .....	+	+	+	±	-	-

+++ = complete precipitation.

++ = almost complete.

+ = many flocculi in suspension.

± = few flocculi in suspension.

- = remains uniformly turbid.

The presence of normal agglutinins, in the serum of Belgian hares, for the hog-cholera bacillus does not nearly equal the amount present for the typhoid bacillus. However, as they appeared to be present in sufficient quantity to obscure the value of the comparative test so far as the hog-cholera bacillus was concerned, the rabbit was given another immunizing dose and another series of tests made in the manner detailed above, excepting that the readings were taken after two hours at 37° C. and the culture from the liver of H. 14 substituted for that of rat 20. The results of this test show not only that the culture from azoia rat 2 and that from the pseudotuberculous disease of guinea-pigs are identical but that they are probably more closely related to *B. enteritidis* H. 14 than to hog-cholera or to Danysz bacillus, though Danysz bacillus is said to be identical with *B. enteritidis* (Gärtner).

TABLE 3.

CULTURES	DILUTIONS OF SERUM						
	$\frac{1}{200}$	$\frac{1}{400}$	$\frac{1}{600}$	$\frac{1}{800}$	$\frac{1}{1000}$	$\frac{1}{2000}$	$\frac{1}{4000}$
Azoo rat 2.....	+++	++	++	+	±	±	±
Guinea-pig, July 13.	+++	++	++	+	±	±	±
Liver, Human 14....	±	—	—	—	—	—	—
Danysz.....	—	—	—	—	—	—	—
Hog cholera.....	—	—	—	—	—	—	—

PATHOGENICITY OF *B. pestis-caviae*.

*Guinea-pigs*.—When guinea-pigs of 300–500 gms. weight are injected subcutaneously with 2 c.c. of a 24-hour-old sugar-free broth culture they die in three to six days. On dissection the skin of the abdomen is firmly bound to the muscular wall by a hemorrhagic fibrinous exudate, which is of a purulent nature at the point of inoculation. A sero-gelatinous exudate may occur in the groins. The spleen is enlarged and soft when the animal lives for five or six days. Hydrothorax and fibrino-purulent pericarditis may be found. The other internal organs are generally congested. Numerous bipolar staining bacilli often in pairs occur in the subcutis or pericardial exudate. A few bipolar-staining diplobacilli may be found in spleen or liver smears. Cultures show that there are numerous bacilli in the spleen even when they appear scarce microscopically. Passage from guinea-pig to guinea-pig produces a marked increase in the severity of the local reaction but does not materially shorten the course of the infection during 11 or 12 passages as detailed further on in Table 4. Doses of 0.25–0.75 c.c. usually produce a localized abscess followed by necrosis, sloughing, and recovery. Intraperitoneal injection produces death more rapidly with marked fibrino-purulent peritonitis and septicemia.

*Rats* (*M. norvegicus*).—Four c.c. of a 24-hour-old sugar-free broth culture must be injected subcutaneously into adult rats (150–350 gms.) in order to produce any uniform lethal effects. When they succumb, the average time appears to be about 8–12 days. On dissection there is local abscess production. The spleen is enlarged, sometimes to four or five times the normal size, soft, and usually shows numerous yellowish-white specks of necrosis. If the rat survives and is chloroformed, minute yellowish-white nodules, 0.5–



2 mm. in size may be found in this organ. The liver may simply appear congested or also show numerous yellowish-white foci of necrosis. Occasionally there are dark coffee-colored areas of hemorrhage in the lungs; also sometimes hydrothorax. The lymph glands do not appear to be involved and I have not seen the secondary pericarditis which sometimes occurs in guinea-pigs.

When ingested, a large percentage of adult rats are immune, though they occasionally die with the above-mentioned anatomical changes or simply with congestion and swelling of the spleen and liver. Young rats (50-150 gms.) and mice (*M. musculus*) are quite uniformly susceptible to infection by feeding. They either succumb rapidly to an acute septicemia without any marked anatomical changes or, when more resistant, they show the anatomical changes in the spleen and liver, and sometimes hemorrhages in the lungs, as described above. Rats infected with this virus do not appear to be ill until shortly before their death. I have on a few occasions watched them die. They lie with legs extended and exhibit embarrassed respiration. The respirations gradually become slower and shallower until death ensues.

Looking back over the feeding experiments with azoas already detailed, this factor of age will explain the difference in the results obtained in Series I and Series II.

*Rabbits.*—A rabbit weighing 1,160 gms. was injected subcutaneously with 1 c.c. of a 24-hour-old broth culture. A localized abscess was produced. This ruptured and healed.

I have not found time to test the possible pathogenicity of *B. pestis-caviae* for the various domestic animals. This has apparently been done by Parke, Davis & Co.

A single feeding-experiment with a monkey will be detailed. An adult Rhesus monkey was starved 12 hours and then fed on 30 c.c. of a 24-hour-old sugar-free broth culture, inoculated directly from the spleen of rat 18 (Table 5) mixed with boiled rice and chopped apple. It ate the mixture greedily and has remained well up to date, a period of one month. The ground squirrel of this region, *O. beecheyi*, is immune to infection by feeding on large doses of sugar-free broth cultures.

Various attempts have been made to raise the virulence of this

organism and these will be detailed here in connection with the general subject of pathogenicity.

EXPERIMENTS ATTEMPTING TO RAISE THE VIRULENCE BY PASSAGE  
FROM RAT TO RAT BY INOCULATION AND FEEDING.

In this series adult rats (*M. norvegicus*) over 150 gms. were used throughout. The original culture of *B. pestis-caviae* from the spleen of the guinea-pig of July 13 was used to start the series on September 2, and all subsequent experimental work has been conducted with this stock or its subcultures. Preliminary experiments showed that it was necessary to use 4 c.c. of a sugar-free broth culture grown for 24 hours at 37° C. in order to get any uniformly lethal effects when injected subcutaneously into adult rats. Occasionally death was produced by 2 or 3 c.c. In order to shorten the time the organism must spend outside of an animal host, 10 c.c. sugar-free broth tubes were inoculated directly from the spleen of one victim and then after 24 hours' growth 4 c.c. were inoculated subcutaneously into another. These fluid cultures were controlled for purity as far as practicable by the examination of hanging-drop preparations and control cultures on agar slants. Occasionally dextrose, lactose, and saccharose tubes were inoculated as controls. Out of four series of such passages I will cite one:

Table 5 records the subcutaneous passages through a series of adult rats. The culture with which the first rat was injected was derived from the spleen of a rat which died on the tenth day after feeding on 7 c.c. of a 24-hour-old milk culture of *B. pestis-caviae*. The results showed at least that no rapid gain in virulence could be obtained by this method.

TABLE 5.

Rat	Days till Death	Chloroformed in Days	Organism Recov- ered in Cultures
2.....	8	..	++
9.....	8	..	+
13.....	8	..	+++
18.....	8	..	+++
32.....	..	20	—

+++ = Numerous colonies, spleen, liver, and heart's blood.

++ = Numerous colonies, spleen only.

+ = Few colonies, spleen.

Table 6 illustrates another series in which passages by inoculation

and feeding were combined. This series was started as indicated above, Table 5. When rat 2 died its spleen and liver were fed to rat 13 and likewise the spleen and liver of rat 13 to rat 14, and cultures from the spleen of rat 14 inoculated into rats 25, 26, 27 with negative results:

TABLE 6.

Rat	Days till Death	Chloroformed in Days	Organism Recovered in Cultures
2.....	8	..	++
13.....	8	..	+++
14.....	16	..	+++
25, 26, 27.....	..	21	—

INOCULATION AND FEEDING EXPERIMENTS FOLLOWING THE ATTEMPT  
TO RAISE THE VIRULENCE OF *B. pestis-caviae* BY  
PASSAGE THROUGH GUINEA-PIGS.

Experiments showed that about 2 c.c. of a 24-hour-old sugar-free broth culture was necessary to kill guinea-pigs of 300–500 gms. when injected subcutaneously. The culture fluid used for passage was inoculated directly from the spleen of a dead guinea-pig. The passages can be divided into two series. (Table 7.)

TABLE 7.

SERIES I				SERIES II			
Guinea-pig Number	Weight Gms.	Dose Subcutaneously in c.c.	Days before Death	Guinea-pig Number	Weight Gms.	Dose Subcutaneously in c.c.	Days before Death
1.....	460	2 (intraperitoneal)	1	1.....	460	2 (intraperitoneal)	1
2.....	475	2	5	2.....	475	2	5
4.....	450	2	3	4.....	450	2	3
5.....	471	2	4	6.....	455	1	7
7.....	514	2	6	9.....	343	0.5	6
11 and 12.....	370	2	4	14.....	390	1	6
	398						
16.....	331	1.5	3	18.....	290	1	4
17.....	300	1.5	2	21.....	495	1	9
19.....	310	1	2	24.....	500	3	3
20.....	530	1.5	3	25.....	420	3	3
22.....	466	0.75	abscess recovered	26.....	435	2	3
				27.....	458	2	6

There was evidently no material increase in the virulence of the organism for guinea-pigs in 11 or 12 such passages. An adult rat was fed on the spleen, liver, and a piece of the local reaction of guinea-pig 12 (Series I, Table 7). It died in six days. The cultures from its spleen were passed through four series of rats by subcutaneous

inoculation. Table 8 details two of these series in which perhaps some initial increase in virulence is shown, as heretofore the subcutaneous injection of the same dose produced death in about eight days.

TABLE 8.

SERIES I				SERIES II			
Rat	Days till Death	Chloroformed in Days	Organism Recovered in Cultures	Rat	Days till Death	Chloroformed in Days	Organism Recovered in Cultures
11.....	4	..	++	11.....	4	..	++
15.....	..	8	+	15.....	..	8	+
20.....	4	..	+++	19.....	5	..	++
				20.....	4	..	+++
28-20.....	..	23	-	21.....	5	..	++
				22.....	..	24	-
				23.....	..	24	-
				24.....	..	24	-

Further, the following feeding experiment shows that a few passages through guinea-pigs do not materially increase the virulence for adult rats: Each of 10 adult rats consumed 20 c.c. of a 24-hour-old sugar-free broth culture, inoculated directly from the spleen of guinea-pig 7 (Fifth passage, Series I, Table 7). They were chloroformed on the twenty-seventh day and found sterile culturally.

#### EXPERIMENTS ATTEMPTING TO RAISE THE VIRULENCE BY FEEDING THE ORGANS OF RATS TO RATS.

These experiments show that adult rats are not suitable for this method of passage. Out of 20 rats fed on the spleen and liver of rats dead after inoculation, 17 were chloroformed and found uninfected in from 18-36 days after the feeding. In one instance the infection was transmitted through two rats but died out in the third as shown in Table 9.

TABLE 9.

Rat	Fed on	Days till Death	Chloroformed	Cultures
2.....	(inoculated)	8	..	++
13.....	spleen and liver 2	8	..	+++
14.....	spleen and liver 13	14	..	+++
21.....	spleen and liver 14	..	22	-

## FEEDING EXPERIMENTS WITH YOUNG RATS AND MICE.

*Young rats (M. norvegicus).*—Seventeen rats (50–75 gms.) were placed together in a large cage and fed on about 50 c.c. of a 24-hour-old sugar-free broth culture inoculated directly from the spleen of rat 18 (Table 5), mixed with boiled rice and rolled oats. Three of them died on the fifth day after feeding; one on the tenth day; three on the eleventh day; one on the twelfth day; one on the fourteenth day; one on the fifteenth day; i. e., 10 of the 17 were dead. Most of them showed characteristic anatomical changes post mortem. Cultures from their spleens and livers showed numerous characteristic colonies and in three instances these colonies were studied in greater detail and corresponded with the organism fed to the rats.

Further the spleens and livers of the four rats which died on the tenth and eleventh days were fed to two young rats. Both of these died on the night of the sixth day after feeding. At the post-mortem examination they both showed spleens enlarged to about four times the normal size and their spleens and livers were full of numerous yellowish-white foci of necrosis. Cultures from their spleens yielded numerous characteristic ring colonies.

A large adult male rat was fed on the body of the young rat which died on the fourteenth day. It was chloroformed in an apparently healthy condition on the sixth day after feeding and showed a typical large spleen full of yellowish granules and a much speckled liver. Of course it is possible that this rat might have subsequently recovered.

*Mice (M. musculus).*—Twenty c.c. of a 24-hour-old broth culture containing 0.1 per cent dextrose, inoculated from an agar culture from the spleen of guinea-pig 26 (Eleventh passage, Series II, Table 7) was mixed with cornmeal and fed to six mice. The mice were in one cage. One died on the eighth day, two on the twelfth day; one on the fourteenth; and one on the seventeenth day, after feeding. They showed typical spleens and livers post mortem. Litmus-lactose-agar plates from the spleen of the mouse which died on the eighth day showed hundreds of blue colonies one of which corresponded culturally with *B. pestis-caviae*. The sixth mouse was still alive but sick on the nineteenth day after the feeding.

ACTION OF THE VIRUS UPON YOUNG WHITE RATS SUCKLING AN  
INFECTED MOTHER.

An adult female white rat (250 gms.) with seven young rats, 10 days old, was fed on rolled oats soaked with 15 c.c. of a 24-hour-old broth culture inoculated directly from the spleen of guinea-pig 20 (Table 7) and then placed back in the cage with her young.

On the tenth day after the mother was fed one of the young rats was found in a dying condition. Four more young were found dead on the thirteenth day. Two young rats were still alive and eating nothing but their mother's milk. These two were found dead on the twentieth and twenty-second days respectively after the feeding. Three of these young rats had been partially devoured by the mother and were not examined. The remaining four showed no particular anatomical changes excepting slight congestion of the spleen. Cultures from the spleens of these four yielded typical colonies on litmus-lactose-agar plates and subcultures proved to be actively motile rods which produced gas from dextrose, but did not alter lactose nor saccharose and were completely agglutinated by the serum of a Belgian hare immunized against culture azoia rat 2 at a dilution of 1:200 in three hours at 37° C.

The mother was chloroformed in an apparently healthy condition on the twenty-second day after the virus had been fed; the mammary glands were completely atrophied: the liver was congested: the spleen was about twice the normal size, soft, and full of fine yellowish-white foci of necrosis. Cultures from the spleen and liver yielded no growth. While the presumptive evidence is that the germs reached the young through their mother's milk, one cannot rule out the possibility that the mother infected her teats after feeding on the virus. The anatomical changes clearly indicate that the mother had had a septicemic infection from which she was recovering.

## SUMMARY AND CONCLUSIONS.

1. *Bacillus pestis-caviae* is an organism belonging to the group of bacteria best represented by the hog-cholera bacillus. So far as is known its original host is the guinea-pig, *Cavia Aperea*, in the spleen of which it produces pseudotuberculous nodules. It was described over 10 years ago by Theobald Smith. Culturally it is identical with

the virus of a commercial ratticide known as azoa, and with Danysz bacillus (Král), with a strain of *B. enteritidis* isolated from the liver of a human case, and with a strain of the hog-cholera bacillus, excepting the lack of indol production in the case of hog-cholera bacillus. Agglutination experiments show that it is identical with the virus of azoa, that it belongs to this group of bacteria and is perhaps more closely, though remotely, related to the members of this group represented by *B. enteritidis*.

2. When ingested it is acutely pathogenic for young rats (*M. norvegicus*) (50–150 gms.) and mice (*M. musculus*) which die of a septicemia accompanied by swelling and congestion of the spleen and liver, with or without the production of multiple yellowish-white foci of necrosis in these organs. The lesions in these organs often closely simulate those of plague but should not cause confusion in the naked-eye diagnosis of plague in rats: whereas hydrothorax is sometimes present in rats killed by this virus, the lungs are pale and further buboes and subcutaneous injection are never present. Bipolar-staining rods, which often occur in pairs, can be found only with difficulty in microscopic preparations from infected rats.

A large percentage of adult rats (150–350 gms.) are naturally immune to infection through the gastro-intestinal tract or when infected they subsequently recover.

3. Passing the organism by subcutaneous inoculation, through 11 or 12 guinea-pigs, does not materially increase its virulence for guinea-pigs though this method may increase its virulence to some extent for rats.

4. Adult rats are unsuitable for experimental passages by inoculation and feeding.

5. All the suckling young of an infected white rat died of the disease.

NOTE.—After this article had been sent in for publication I noted that the last Indian Plague Commission undoubtedly encountered this bacillus as the cause of an epizootic among their young stock guinea-pigs and also occasionally in rats. They placed it in the *B. enteritidis* group (*Jour. Hyg.*, 1908, 8, p. 306).

## EXPERIMENTS ON THE GERMICIDAL ACTION OF COW'S MILK.\*

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Since Fokker<sup>1</sup> suggested that fresh cow's milk possessed a germicidal action, the subject has been investigated by many observers and the conclusions arrived at are by no means harmonious. One faction claims that there is no germicidal action, the chief workers being Moro,<sup>2</sup> Honigman,<sup>3</sup> Hesse,<sup>4</sup> Basenau,<sup>5</sup> and Stocking.<sup>6</sup> The opposite opinion has been advocated by Ehrlich and Brieger<sup>7</sup> who found that antitoxic and bactericidal substances are transferred by means of milk. Among others who have defended the presence of germicidal substances in fresh milk are Park,<sup>8</sup> Hunziker,<sup>9</sup> Koning,<sup>10</sup> Kolle,<sup>11</sup> and Hippus.<sup>12</sup> The literature has been discussed frequently and thoroughly, so that it seems unnecessary to take up that phase of the subject here.

Experiments made by one of us, the results of which were reported at the meeting of American Bacteriologists in New York in 1906, seemed to show that the germicidal action is at least quite variable, that some bacteria decrease in numbers for several hours; others, however, hold their own, while some even increase slightly from the start. It cannot be denied that there is some restraining action in fresh milk, although the decrease never compares in degree with the germicidal action exerted by blood or blood serum. The results of the work referred to were obtained by inoculating bacteria in suspension into the milk and species were selected chiefly which are known to multiply readily in sterilized milk. It was shown that the objection that many

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<sup>1</sup> *Ztschr. f. Hyg.*, 1894, 17, p. 238.

<sup>2</sup> *Ztschr. f. Hyg.*, 1890, 9, p. 41.

<sup>3</sup> *Archiv f. Hyg.*, 1895, 23, p. 170.

<sup>4</sup> *Mönc. med. Wchnschr.*, 1891, 48, p. 1770.

<sup>5</sup> *Storrs Agric. Exper. Station Report*, 1904, p. 89.

<sup>6</sup> *Ztschr. f. Hyg.*, 1893, 14, p. 207.

<sup>7</sup> *Ztschr. f. Hyg.*, 1893, 13, p. 336.

<sup>8</sup> *N. Y. University Bull. Med. Sc.*, 1901, 1, p. 2.

<sup>9</sup> *Cornell Univ. Agric. Expr. Station Bull.*, 1901, No. 197.

<sup>10</sup> *Milchwirthsch. Zentralbl.*, 1905, 1, p. 49.

<sup>11</sup> *Milch-hygieneische Untersuchungen*, Jena, 1904, G. Fischer.

<sup>12</sup> *Jahrbuch f. Kinderheilkunde*, 1905, 11, p. 365.



bacteria which gain access to milk, find milk an unsuitable medium and die, cannot be upheld.

Lately Rosenau and McCoy<sup>1</sup> published experiments which suggest that there is no actual decrease in any case in fresh milk, but that the apparent decrease is due to the presence of agglutinins, which cause the bacteria to clump so as to form a smaller number of colonies as the time progresses. If this be true it is evident that milk serum should agglutinate bacteria in suspension.

The work of Rosenau and McCoy suggested to us a plan of work which was carried out and which it is the purpose of this paper to discuss.

Some preliminary experiments with milk serum made several years ago showed that it actually is capable of agglutinating many species of bacteria, occasionally in dilutions higher than 1:200. The serum was obtained by passing milk through a Berkefeld filter and the filtrate was mixed with suspensions of bacteria in physiological salt solution. The same method was followed in the present experiments and in addition milk was inoculated with suspensions of the identical cultures used for the agglutination tests. The milk was obtained fresh from three cows alternately. The udders were previously washed with a solution of bichloride of mercury and kept covered with a cloth moistened with the same solution during milking. Sterilized wide-mouth glass-stoppered bottles of 250 c.c. capacity served as receptacles. Of this milk 10 c.c. were distributed into each of 10 sterilized culture tubes and the balance of the milk poured on a sterilized Berkefeld filter and the filtrate tested for the presence of agglutinins.

The tubes were treated in the following manner:

One c.c. of each of two tubes was diluted with 99 c.c. of sterilized water and plated. One of these dilutions was shaken moderately, the other vigorously, before plating. Two tubes were then inoculated with a suspension of some organism and a dilution of 1:10,000 prepared from each and plated. One of these tubes was shaken moderately, the other vigorously. Two tubes were then heated for 30 minutes at 56° C., another set of two tubes at 75° C., and a third set at 100°. Each pair after cooling was inoculated with the same organism with which the raw milk had been inoculated and plates prepared from dilutions of 1:10,000, one of each pair shaken moderately, the other vigorously. All tubes were then placed in an incubator at 37° C. and similar

<sup>1</sup> *Jour. Exper. Med.*, 1908, 18, p. 165.

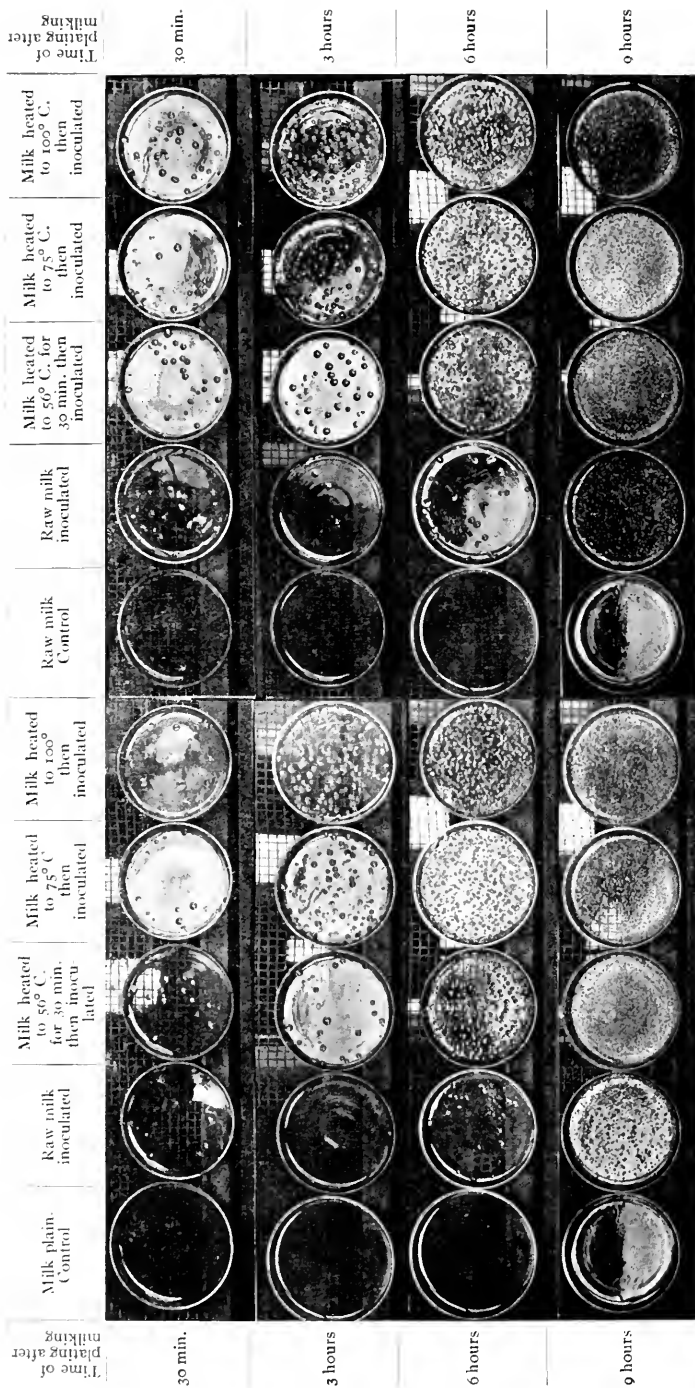


FIG. 1.—Action of cow's milk, raw and heated to various temperatures, on *B. mucosus*.

plates prepared from each tube at intervals of two hours. The plates remained in the incubator at 37° C. for three days when the colonies were counted.

As soon as 6 c.c. or more of filtrate were obtained from the milk filtering through the Berkefeld, the test for agglutination was commenced. Small tubes of even caliber were sterilized and dilutions of the serum prepared so that each tube, after adding the suspension of bacteria, contained 4 c.c. The dilutions prepared were 1:2, 1:5, 1:10, 1:20, 1:50, 1:100, 1:200, 1:500, and 1:1,000. Observations were taken after 2, 4, and 20 hours. It was found that dilutions of 1:2 and 1:5 contained so much milk serum, that the bacteria multiplied too rapidly and agglutination could not be recognized. In all cases, excepting with *B. coli*, there was no appreciable agglutination within the first four hours and we have therefore omitted these results from the table. No agglutination was observed in dilutions of 1:1,000, and only in one case 1:500. The suspension of bacteria employed in the agglutination tests was prepared from the same 24-hour agar culture which was inoculated into the milk for plating.

Several points of interest can be recorded by a detailed study of the table of results appended:

1. By adding the figures and calculating averages, we find that, although there is a decrease of numbers of bacteria up to two hours and a half, and even to four hours and a half in some samples of milk, this decrease is counterbalanced by an increase in the number in other samples, so that a curve, as shown in the accompanying chart, shows a slight increase. This increase is more pronounced after four hours and a half and becomes still more so after six hours and a half and eight hours and a half. The average number of bacteria in the inoculated milk, however, shows a decided decrease for at least two hours and a half, after which period there is a gradual increase. It must be remembered in this connection that by keeping the milk at 37° C. the so-called germicidal action is of shorter duration than if the milk is kept at a lower temperature.

The milk heated to 56° C. and inoculated with bacterial suspensions shows a steadily ascending curve, if the average is taken. This curve rises but slowly for the first two hours and a half, but quite rapidly after four hours and a half. The average number of bacteria in the milk heated to 75° C. and 100° C. rises more rapidly than in the milk heated to 56° C. for the first two hours and a half, and the curve ascends very decidedly after this period. The difference in the curves of the numbers of bacteria at 75° C. and 100° C. is slight and but one curve has been plotted on the chart.

2. The differences in counts of bacteria from milk shaken moderately and milk shaken vigorously are quite insignificant, the only

appreciable difference being found in the cases of *B. pyocyaneus* in raw milk and *B. prodigiosus* in milk heated to 56° C. *B. pyocyaneus* increases if shaken vigorously, but decreases if shaken moderately.

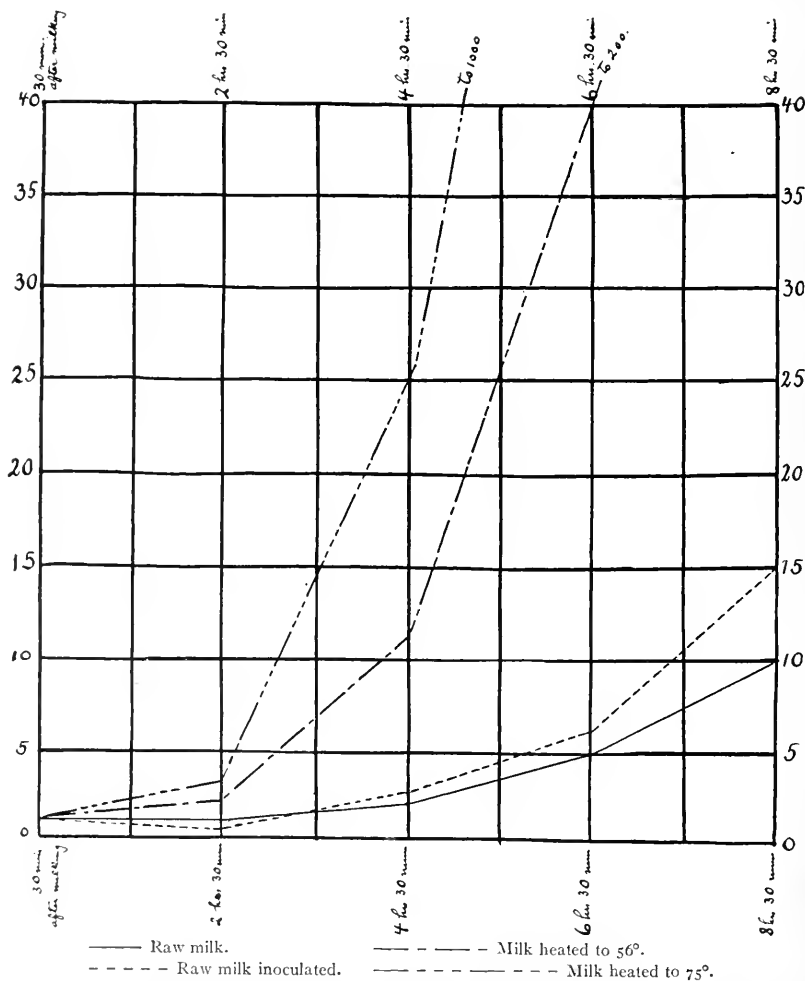


CHART I.—Relative Growth of Bacteria in Raw Milk, Raw Milk Inoculated, and Inoculated Milk, Previously Heated to 56° and 75° C.

*Sp. cholerae* decreases in both, but the decrease is more pronounced if shaken moderately. These results do not exclude the possibility, that we might have found more difference if the vigorous shaking

had been done by means of some mechanical device rather than by hand. When moderately shaken the flask was rotated gently until the mixture was homogeneous; when vigorously shaken the flask was shaken 50 to 75 times.

3. If we study the decrease or increase of the various species individually we find that in raw milk *B. coli*, *B. pyocyaneus*, *B. aërogenes*, *B. mucosus*, *B. dysenteriae*, and *Staph. aureus* decrease in numbers for at least two hours and a half, but that there is a steady increase after four hours and a half. *Bacillus No. 2* from milk, *B. cloacae*, *B. cholerae-suis*, and *B. typhosus* decrease for at least two hours and a half with an increase after six hours and a half. *B. prodigiosus* and *Sp. cholerae* decrease for at least six hours and a half, and *Sarcina lutea* stands in the isolated position of showing a decrease after eight hours and a half. When heating the milk to 56° C. for 30 minutes the *Bacillus No. 2* from milk decreases for at least two hours and a half after which time there is a decided increase, and *Sarcina lutea* decreases for at least eight hours and a half. In the milk heated to 75° C. we have an increase of numbers in all species excepting *Sarcina lutea* from the beginning. This organism decreases for two hours and a half before an increase is noted. Finally *Strept. lacticus* occupies the the unique position of increasing from the start even in raw milk.

4. The point of chief interest brought out by this work is the relation of agglutination of certain bacteria by milk serum to the decrease of numbers of the same species. The results are consistent on the whole, although there are some exceptions. It must be remembered, however, that milk is rather difficult to filter through a Berkefeld filter and we have found that the time required for filtering varies greatly with different milks and different filters. In some instances we obtained enough serum after an hour and a half; in others eight hours were required to yield enough serum to work with. It is to be presumed that, as the time for filtration is prolonged, the agglutinins, which may be present in the milk, are active and are consequently consumed, so that the tube reaction becomes less marked in proportion to the time required for filtration.

Good agglutination results were obtained with *B. coli*, *B. aërogenes*, *B. mucosus*, *Staph. aureus*, *Sp. cholerae*, and *B. typhosus*. All

these organisms excepting *B. typhosus* showed a decided decrease in numbers, so that the agglutination phenomenon accounts for this decrease satisfactorily. *Bacillus* No. 2 from milk decreased markedly, although the agglutination reaction was insignificant. Unfortunately our records do not show the time consumed in obtaining a sufficient amount of serum when working with *B. typhosus* and *Bacillus* No. 2 from milk.

*B. prodigiosus*, *Bacillus* No. 1 from milk, and *B. pyocyaneus* decreased but moderately, and in harmony with this fact is that of the moderate agglutination of these organisms. Enough serum was obtained in two or three hours for testing these cultures. *B. cloacae* also decreased moderately. It required five hours to obtain enough serum and the agglutination was wholly negative. *B. chol.-suis* decreased considerably, and it took six hours to obtain enough serum to work with. The agglutination was quite moderate. *Sarcina lutea* decreased considerably, although the agglutination was but fair. In this case the filtering process lasted for five hours.

Milk serum seems to possess an exceptionally marked agglutinative influence on *Sp. cholerae*. This organism agglutinated partially in a dilution of 1:500 and completely in a dilution 1:200. The serum was obtained in an hour and a half. The decrease of this organism in milk is more decided than that of any other species used in the experiments. This observation seems to agree with a statement made by Hesse<sup>1</sup> who found that milk is a poor medium for cholera spirilla and that they died before sufficient acid was formed in the milk to affect them.

We find an exceptional phenomenon with *Strept. lacticus*, which increases markedly from the outset even in raw milk and at the same time is agglutinated considerably. It is difficult to form a theory to explain this. *Strept. lacticus* is usually found in but small numbers in fresh milk and it is possible that it multiplies with such rapidity that the agglutination of the milk is not sufficient to effect a visible decrease. This theory is supported somewhat by the fact that the increase is considerably slower in raw milk than in milk heated to 56° C., and the difference is still more pronounced in milk heated to 75° or 100° C. It has also been shown<sup>2</sup> that *Strept. lacticus* increases

<sup>1</sup> *Loc. cit.*

<sup>2</sup> Heinemann, *Jour. of Infect. Dis.*, 1906, 3, p. 192.

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# A CONTRIBUTION TO THE STUDY OF PYOCYANEUS INFECTIONS WITH A REPORT OF TWO RARE CASES.\*

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IN reviewing the literature of pyocyaneus infections, one is at once struck with the increased frequency with which the germ is encountered, either alone or in association with other organisms, since the introduction of improved methods in the bacteriological examination of material from pathological lesions, has been more widely adopted. The pyocyaneus bacillus has been found in pure culture in all parts of the body, in localized lesions and in general systemic infections. In not a few instances it has been isolated in pure culture from the blood during life. By some it is believed that when this germ is found it is not the cause of the original lesion but that it makes its appearance only after some other organism, having brought about a lowered tissue resistance, has prepared the soil for it and has then disappeared. This explanation may be tenable in many instances but there are others where there can be very little doubt that *B. pyocyaneus* is the primary and sole cause of the lesion.

## INFECTIONS OF THE EAR.

Certain parts of the body seem to be attacked with greater frequency than others, the middle ear appearing to be especially prone to such infections. Sometimes from this focus, which is localized in the beginning, there is an extension to the meninges or a general invasion of the body may take place.

In acute otitis media I have collected from the literature 21 instances of pure infection and 15 of mixed. A few more have been recorded, but I was unable to verify the reports from the original sources.

Among the earlier investigators who have reported the presence of the *B. pyocyaneus*, either in pure culture or in association with other organisms in otitis media, mention should be made of Babes.<sup>1</sup>

\* Received for publication July 20, 1908.



Bloxall<sup>2</sup> reports one case of acute otitis media following scarlet fever which was due to this germ. Chambers,<sup>3</sup> in the examination of the pus from 58 cases of otitis media purulenta, found it present in pure culture six times, twice associated with *Strept. pyogenes*, and twice with *Strept. pyogenes* and *Staph. aureus*. The discharges were profuse and fetid and if imprisoned had a green color. Danilovič<sup>4</sup> and Finkelstein<sup>5</sup> have isolated it and Funke<sup>6</sup> obtained it twice in pure culture and eight times in association with other bacteria in the examination of the discharges from 76 cases of otitis media. Green<sup>7</sup> in the examination of 73 cases of pure infection in acute sup-puration of the tympanum met with it three times; in 28 cases of mixed infection it was present once in association with *Staph. aureus*. In 112 cases of mastoiditis in which a pure culture was present it was found eight times. Gruber<sup>8</sup> and Harbitz<sup>9</sup> have reported its presence, and Hasslauer<sup>10</sup> records 17 cases of primary middle ear disease with spontaneous perforation in which it was the specific cause twice. Helmann<sup>11</sup> found it in pure culture in three cases of otitis externa crouposa. In Horder's<sup>12</sup> case of chronic bilateral otitis media with perforated tympanum, which had existed for several years, there was an acute exacerbation which lasted for five weeks, terminating in paraplegia and death. From the pus in both middle ears, small abscesses in the lungs, and from the pus in the meninges extending from the mid-dorsal region to the cauda equina it was obtained in pure culture, but not from the spleen or blood. No other organism was present in any material examined. Kanthack<sup>13</sup> reports nine cases of chronic otitis in which it was usually encountered with other bacteria. Of the bacilli present only *B. pyocyaneus* was pathogenic. Kossel<sup>14</sup> investigated bacteriologically 38 cases of inflammation of the middle ear post mortem. Three times *B. pyocyaneus* was present in pure culture, once in association with a pseudo-influenza bacillus. In one of these cases there was a severe gastritis, the stomach contents showing this germ in large numbers. The feces were stained a characteristic green. Lermoyez<sup>15</sup> isolated *B. pyocyaneus* in pure culture from a case of perichondritis of the ear muscle. This bacillus proved to be pathogenic to rabbits. Leutert<sup>16</sup> in all his cases of perichondritis of the ear, four in number, found this organism present in pure culture. Maggiora and Gradenigo<sup>17</sup>

found it associated with *Staph. albus* in small numbers in a furuncle of the external auditory canal. They believed that *B. pyocyaneus* was the cause of the trouble. Martha<sup>18</sup> encountered it twice in pure culture in the examination of 53 cases of purulent otitis media. Pes and Gradenigo<sup>19</sup> isolated it twice in pure culture from 15 cases of acute purulent otitis media. Rohrer<sup>20</sup> also records its presence in the discharge from an acute purulent otitis media which had ruptured spontaneously. Ruprecht<sup>21</sup> describes a case of otitis externa crouposa from which on the fifth day of the membrane formation a pure culture of *pyocyaneus* was obtained. Tatsusaburo<sup>22</sup> adds another to the list of cases of perichondritis of the external ear in which this organism was present in pure culture. Voss<sup>23</sup> has contributed to the literature of *pyocyaneus* infections of the ear.

#### INFECTIONS OF THE EYE.

Among those who have isolated this organism in infections of the eye are de Berardinis<sup>24</sup> and Derby.<sup>25</sup> Hanke<sup>26</sup> has reported a case of ring abscess of the cornea from which he isolated an organism belonging in all probability to the *pyocyaneus* group. It was pathogenic for mice and infection of the anterior chamber of the eye of a rabbit produced a typical ring abscess. Happe<sup>27</sup> obtained it in pure culture from a corneal ulcer and injected it into the center of the cornea of a guinea-pig. An *ulcus serpens* developed with an accompanying ring abscess. MacNab<sup>28</sup> reports one case with pure infection in a woman 40 years old. *B. pyocyaneus* was isolated from a round ulcer of the left eye and from the pus in the anterior chamber. Injections of cultures of this organism into a guinea-pig and two rabbits showed that it was highly virulent. It was also obtained from the cornea in an experimental infection which presented the clinical picture of an *ulcus serpens*. Sattler's<sup>29</sup> two cases of panophthalmitis resulted from an injury to the eye in each instance. The first occurred in a stone-cutter 40 years of age and the second in a woman who injured the cornea while splitting wood. Schmidt,<sup>30</sup> in a bacteriological investigation of 30 cases of circumscribed corneal ulcer, encountered it only once in pure culture. In Szczybalski's<sup>31</sup> case nearly all the corneal surface was involved. Iritis and hypopyon

were complications. The infection followed an injury from straw dust. The only organism found was *B. pyocyaneus*.

#### INFECTIONS OF THE RESPIRATORY TRACT.

Barker<sup>32</sup> has reported the presence of *B. pyocyaneus* mixed with other organisms in bronchiectasis. In a case of broncho-pneumonia he encountered it in the pneumonic exudate in pure culture. This infection had developed in a man who died from carcinoma, the primary focus being in the lip with extension to the mouth, pharynx, lung, and bone, and with metastases in the cervical and axillary lymph glands. Blumer<sup>33</sup> isolated it in almost pure culture from an acute angina which clinically resembled diphtheria. Hirschler and Terray<sup>34</sup> found it associated with cocci in a case of gangrene of the lung. Monnier<sup>35</sup> cultivated it from foci of broncho-pneumonia, and from heart blood and pleural exudate. Nabarro<sup>36</sup> describes a case of gangrene of the nose in which it was present. Soltmann's<sup>37</sup> case is particularly interesting since it occurred in a child 13 years old who had been well previous to this infection. Death occurred on the eighth day. From the hepatized areas of the lung, cultures were made which revealed only *B. pyocyaneus*. It was also found in the stomach and intestines. Its presence, in association with other bacteria in the respiratory tract, has been recorded by Stein.<sup>38</sup>

#### INFECTIONS OF THE DIGESTIVE TRACT.

In lesions of the digestive tract it has been found in carious teeth by Arkövy;<sup>39</sup> in lesions of the esophagus, in association with other organisms, by Barker.<sup>40</sup> Booker,<sup>41</sup> in only a few instances, found it in the examination of the intestinal contents of infants suffering from summer diarrheas. Calmette<sup>42</sup> has recorded an interesting investigation carried out in Cochin China to determine the cause of dysentery existing there. In 15 of 16 cases where cultures were made *B. pyocyaneus* was present in the intestinal contents and ulcers in enormous numbers. Practically no other organism grew on the plates. In some instances pure cultures were isolated from the blood. Since Calmette frequently found streptococci associated with the *B. pyocyaneus*, he is inclined to believe that it was their presence in the intestinal contents which predisposed to the pyocyanus infection. In the light of more recent reports it seems very probable that, in

some instances at least, *B. pyocyaneus* may give rise to severe intestinal lesions without the aid of any other organisms. Cook<sup>43</sup> has found that this germ is sometimes the cause of pulp gangrene. Cooley<sup>44</sup> has recently made an interesting contribution to the literature of epidemics caused by *B. pyocyaneus*. He records 28 cases of infantile diarrhea occurring in a hospital, in children ranging in age from a few weeks to 2½ years. The epidemic occurred only in bottle-fed children. The infection was probably transmitted through the milk, which was apparently contaminated from the carelessness of those who attended the babies. Cultures were made from the stools of nine typical cases. In six of these the pyocyaneus bacillus was the predominating organism. In the other three an organism similar in all other respects to that encountered in the first six, except that no pigment formation took place, was isolated. Nine deaths occurred, six of them in infirmiry babies. Of the remaining three one died in convulsions attendant on an acute otitis media, another was only four weeks old, and the third had a prolonged attack with several relapses. Coyne and Hobbs<sup>45</sup> isolated from the contents of the appendix and periappendicular pus in a case of appendicitis *B. pyocyaneus* and *B. coli*. Cultures of the former killed rabbits in 26 hours. The colon germ was not pathogenic for this animal. Czerny and Moser,<sup>46</sup> and Escherich<sup>47</sup> contribute to pyocyaneus infections in nursing infants. Escherich observed in the children's clinic at Graz, immediately following a case of endocarditis and septicemia caused by this organism, two cases of diarrhea in children. In both cases this germ was isolated from the stools. In one case it was found in an abscess of the thigh. Death occurred and in the foci of bronchopneumonia which were present the germ was again encountered. Jakowski<sup>48</sup> isolated it once from a fistula of the small intestine in pure culture and once in large numbers from a fistula of the colon. Kelly<sup>49</sup> in the post-mortem examination of 94 cases of acute appendicitis found the pyocyaneus bacillus present in pure culture six times. In 107 chronic cases it was found only once in pure culture. Lartigau<sup>50</sup> reported an interesting epidemic of dysentery which occurred in 1897. Fifteen persons in all were affected. *B. pyocyaneus* was isolated from the wells from which they obtained their domestic water supply and from the dejecta of the patients. Of the 15 persons affected

four died, one being an adult of 35 years, the remaining three children ranging in age from 19 months to five years. Three of these deaths occurred in one family, seven in all being infected. The one death occurring in the remaining eight cases was in a child three years old. Maggiora<sup>51</sup> examined the stools of 11 persons suffering from a severe epidemic of dysentery. The pyocyaneus bacillus was found five times. Monnier<sup>52</sup> has isolated it in a case of noma. Pottien's<sup>53</sup> three cases of cholera nostras are probably instances of pyocyaneus infection. However, *B. coli* was also present. They were all rapidly fatal. Salus<sup>54</sup> obtained this germ in pure culture, in a woman 26 years old, from a fecal fistula which had resulted from the breaking of adhesions of the intestine to an abdominal tumor of the ovary, which was removed. Thiercelin and Lesage<sup>55</sup> have found it in the intestinal canal in nursing infants with fever. Wollstein<sup>56</sup> has found it in the stomach in ulcerative gastritis.

The epidemics of dysentery described by Calmette, Cooley, and Lartigau offer some strongly convincing evidence that dysentery may be due to the pyocyaneus bacillus without the aid of other organisms as predisposing factors. In addition it is shown that these infections are not confined to young children, though of more frequent occurrence in them than in the adult.

#### INFECTIONS OF SEROUS MEMBRANES.

Barker<sup>57</sup> found, in the systematic bacteriological examination of 800 autopsies at Johns Hopkins Hospital, that the pyocyaneus bacillus was the cause of the infection in only 11 instances. Since it has been so infrequently found when systematic bacteriological examinations of intestinal contents are made, and as it is rarely present post mortem, its presence in lesions, though it be isolated post mortem, cannot be satisfactorily explained by attributing its presence to invasion after death. Of the 11 instances recorded above it was present once in a general peritonitis in a male aged 20. Death followed an operation, the pyocyaneus bacillus and the colon bacillus being present in the peritoneal exudate and on the intestines. From a mesenteric lymph gland *B. pyocyaneus* was obtained in pure culture. It was isolated once in this series from the pericardium and once in an acute endocarditis complicated with general systemic invasion. Blum's<sup>58</sup> case was

that of a child  $2\frac{1}{2}$  years old. During life the bacillus was obtained in pure culture from the blood and post mortem from the heart blood and from the valves, which presented the lesions of verrucous endocarditis. Ernst<sup>59</sup> describes a bacillus which is probably only a variety of *B. pyocyaneus*. This organism was isolated in pure culture from the fluid removed at two different tapplings in a case of tuberculous pericarditis. In the examination post mortem of 12 cases of peritonitis Flexner<sup>60</sup> found 10 in which there was infection. Two were found to be sterile. Of the 10 from which cultures were obtained nine were pure, one mixed. In one case the pyocyaneus bacillus was present in pure culture. In 34 cases of exogenous peritonitis it was found twice. In 60 cases of endogenous peritonitis two were sterile. Of the other 58, 37 were mixed, 21 pure. In this class the *B. pyocyaneus* was encountered three times. Kraft<sup>61</sup> investigated eight cases of acute general peritonitis. In one of these he obtained the *B. pyocyaneus*. Lartigau<sup>62</sup> isolated it in pure culture from the laparotomy wound of an acute peritonitis, and from the blood. He also obtained it from the purulent peritoneal contents in association with a few colon bacilli. In a case of acute pleuritis it was isolated in pure culture. Pakes<sup>63</sup> found pyocyaneus bacillus only once in the examination of 21 cases of ulcerative endocarditis. Perkins<sup>64</sup> grew it in pure culture from the peritoneal exudate in a fibrino-purulent peritonitis which developed after a gastro-enterostomy following the removal of a scirrhus carcinoma of the pylorus. Pevzner<sup>65</sup> in the examination of 18 cases of acute endocarditis encountered it once associated with Fraenkel's diplococcus.

#### INFECTIONS OF THE MENINGES.

B rka<sup>66</sup> obtained post mortem a virulent culture of the pyocyaneus bacillus from the yellow purulent deposit in the meninges of an adult. The point of entrance could not be determined, both middle ears being sound. Councilman<sup>67</sup> encountered it in association with the *Staph. aureus*. Kossel<sup>68</sup> cultivated it from the pus of the meninges of a child six weeks old. It was obtained in pure culture from the pia and from the blood in the heart. Since it was found in the pus from the middle ear it is quite probable that this was the primary focus of infection. Pěšina and Honl<sup>69</sup> obtained it in association with Friedländer's bacillus in a case of purulent meningitis in an adult. Since

the disease process developed with enormous rapidity they believe that it was due to the combined action of the two organisms.

#### INFECTIONS OF THE GENITO-URINARY TRACT.

Barker<sup>70</sup> has found this germ associated with other organisms in both acute and chronic cystitis. Bernhardt<sup>71</sup> reports a case of pyelo-nephritis with infection of the epididymis and prostate in a man 53 years old. Blumer and Lartigau<sup>72</sup> found it with *B. proteus vulgaris* in three cases of ascending urinary infection. In 26 cases of acute cystitis examined by Brown<sup>73</sup> *B. pyocyaneus* was present once in pure culture. Charrin<sup>74</sup> found it associated with a few streptococci in a case of puerperal fever. Hirschberg<sup>75</sup> grew in pure culture from the foul-smelling pus in a case of orchitis and periorchitis *B. pyocyaneus* (a). Jatkewitsch<sup>76</sup> cultivated it from the urine of a man afflicted with ulcers which also were probably due to this germ, though no cultures were made from the green pus from the ulcers. Laplace<sup>77</sup> encountered it in six cases of endocervicitis in association with cocci. Le Noir<sup>78</sup> has met with it in urinary infections. McWeeney<sup>79</sup> grew it from purulent urine and Motz<sup>80</sup> records its presence in pathological urine. Oettinger<sup>81</sup> found it in the urine in a case in which it was secured from the hemorrhagic vesicles on the thigh and surrounding parts in pure culture. Perkins<sup>82</sup> records three interesting cases of infection of the genital tract. One of these was a case of puerperal septicemia in which *B. pyocyaneus* was found in pure culture in the cerebro-spinal exudate and in the liver. In the uterus staphylococci were also present. In a second case with a complicating broncho-pneumonia it was found in pure culture in the uterus, and in association with a few large unidentified bacilli in the lungs. He also isolated it from the testicle in pure culture in a case of acute orchitis.

#### INFECTIONS OF THE SKIN.

Adamson<sup>83</sup> has reported the presence of *B. pyocyaneus* in association with streptococci and staphylococci not only in the lesions but also in the heart blood and in sections of the involved tissue. Burot<sup>84</sup> found it in an infection of the skin with widespread ulceration which developed in the course of a severe disease. Muscular atrophy, paralysis, and contractures developed during the course of the disease. The bacillus was found in the pus from the ulcers, in the sanguinolent

secretions of the nose, but it could not be obtained from the blood. Hitschmann and Kreibich<sup>85</sup> isolated it in pure culture from two cases of ecthyma gangrenosum with hemorrhagic diathesis and enteritis. It was obtained from the diseased parts of the skin and from hemorrhagic areas in the internal organs. In a later contribution they report another case due to the same organism. Perkins<sup>86</sup> found it in pure culture in a pustular eruption of the inner surfaces of the thighs. This eruption was of the ecthymatous type. Pernet<sup>87</sup> obtained pure cultures of pyocyaneus from the lesions of pemphigus vegetans. Symmers<sup>88</sup> reports a chromogenic micro-organism found in the vesicles of herpes labialis which probably belongs to the pyocyaneus group.

It seems probable that some of the lesions reported as localized skin lesions are only local manifestations of a general infection. In cases where the symptoms are especially severe the probabilities of a general invasion of the body are correspondingly increased.

#### INFECTIONS OF A MORE GENERAL DISTRIBUTION.

Babes<sup>89</sup> isolated *B. pyocyaneus* from a post-scarlatinous phlegmon. In the organs streptococci were also present. Benfey<sup>90</sup> obtained it from the amber-colored fluid secured by lumbar puncture from a child eight days old. Cultures were made from the spinal fluid, which was withdrawn on account of convulsions, two days before death, and from the brain, pericardial fluid, heart blood, and spleen. In every instance the bacillus was present in pure culture. Boinet<sup>91</sup> obtained it from the blood 24 hours before death in pure culture in a young woman suffering from purulent articular gonorrheal infection. Brill and Libman<sup>92</sup> in a case of septicemia found at first staphylococci. The patient, who was 23 years old, was apparently getting better. This period of improvement was followed by severe septic symptoms accompanied by bronze-colored changes in the skin. Two days before death 6 c.c. of blood were obtained by venous puncture. Cultures made from this blood revealed only *B. pyocyaneus*. Post mortem pyocyaneus was found in pure cultures in the liver, spleen, and kidney. Sections from the organs showed this germ present in large numbers, the capillaries being plugged almost full. In this case the evidence is conclusive of a general pyocyaneus infection occurring in an adult during life. De la Camp<sup>93</sup> contributes a case in a woman of 51



with all the signs of a chronic sepsis. The disease began with pains in the joints. This was followed by a rhinitis with headache. Later fever appeared with purpuric spots and an infiltration of the legs and feet. From the fluid removed from the infiltrated areas during life the pyocyaneus bacillus was obtained in pure culture. Five-tenths of a c.c. of a broth culture grown from the fluid killed a guinea-pig in 14 hours. In the heart blood and in the exudates the same germ was present. Post mortem it was grown from heart blood, verrucous deposits on the mitral valves, and from the spleen. Charrin<sup>94</sup> describes two cases of an infectious process occurring in a brother and sister in which the pyocyaneus bacillus was found. Clinically there were fever, diarrhea, and albuminuria, the symptoms resembling those of typhoid fever or cerebro-spinal meningitis. On the eleventh or twelfth day a vesicular eruption appeared in which this organism was present. One of the children died and from the heart blood the *B. pyocyaneus* was obtained in pure culture. Eastman and Keene<sup>95</sup> report a case of pyocyaneus septicemia in a girl 17 years of age who recovered from her infection. The germ was found in the blood in numerous cultures made from various parts of the body at different intervals. No other germ was present in the cultures. Infection was probably local at first, its primary focus having apparently arisen in a blastomycetic lesion of the skin. Ehlers<sup>96</sup> describes two cases in children, a brother and sister, aged respectively 11 and 12. After a prodromal period there was an elevation of temperature, profuse diarrhea, enlargement of the spleen, mental depression, and prostration. The symptoms suggested typhoid fever or cerebro-spinal meningitis. In about 12 days a papular eruption appeared on the anterior surface of the body and limbs. This eruption became pustular, resembling ecthyma in appearance. The contents of the pustules were blue in color. Ulcers formed with hard borders which were pigmented by hemorrhages. One of the children died with a severe enteritis. From the hemorrhagic pustules and the heart blood the pyocyaneus bacillus was obtained in pure culture seven hours after death. Finkelstein<sup>97</sup> has contributed four cases of pyocyaneus infection in young children. Three of these children suffered from hemorrhagic diatheses during the last few days of their illness. The course of the disease in all three cases was long continued and

exhausting. Two of them had otitis media. Besides other organisms *B. pyocyaneus* was found in the organs and blood post mortem. Finkelstein thinks that these infections are not to be regarded as the original cause of the trouble but merely represent a terminal infection which was due to the loss of the normal protective powers of the body. In a fourth case, in a child three months old, Finkelstein obtained from a vein two days before death 2 c.c. of blood. From cultures of this blood he isolated the pyocyaneus bacillus. Fraenkel<sup>98</sup> describes four cases of infection which he thinks were all due to the pyocyaneus bacillus. From Case 1 it was not obtained in culture. In Case 2 it was found in pure culture in pus from the ear, in the heart blood, and in foci in the kidney. In Case 3 it was found in pus from the ear in association with Fraenkel's diplococcus. From the heart blood pure cultures were obtained. In Case 4 the heart blood contained pyocyaneus in pure culture. Huebener's<sup>99</sup> case is of especial interest since it occurred in a man of strong physique who up to the time of his illness had been completely sound. The original lesion was apparently a circumscribed suppurative focus in the region of the sacrum which had extended from the intestine. A general septicemia ensued with metastases in the meninges and kidneys. There was an almost complete suppression of the bowel movement and of the secretion of urine. The pyocyaneus bacillus was present in the purulent foci, in several blood cultures obtained by venous puncture, in the urine in enormous numbers, and in small numbers in the sputum. These cultures were highly virulent for guinea-pigs. Streaks from the liver, kidney, spleen, and heart blood revealed staphylococci in addition. Numerous sections of the involved tissue of the organs showed nearly always smaller or larger numbers of *B. pyocyaneus*. Karlinski<sup>100</sup> reports a generalized infection in which the germ was obtained from the skin vesicles, spleen, blood, and Peyer's patches in pure culture. In a phlegmon of the forearm it was found in association with *Staph. aureus*, the latter organism being found nowhere else. Kossel<sup>101</sup> contributes three additional cases. In the first, which occurred in an atrophic child six weeks old, the pyocyaneus bacillus was obtained in pure culture from the purulent exudate of the pia and from the heart blood. Pus from the ear and fluid from the edematous foci in the lungs contained

both the pyocyaneus bacillus and Fraenkel's germ. In the second case, in a rachitic child of two years suffering from measles, death occurred at the end of five days. The illness was characterized by high temperature and symptoms of broncho-pneumonia. In the pus from the ear, from the antrum of Highmore, and the nasopharynx only *B. pyocyaneus* was grown. In the middle ear itself a few diplococci were found. The third case was in a child four weeks old, illness being ushered in with diarrhea, loss of appetite, and great weakness. The diapers became dark green on exposure to air and the stools were also dark green. There were many pyocyaneus bacilli present in the feces. Most of the common intestinal organisms had been displaced. After much vomiting and many green bowel-evacuations, which rapidly exhausted the child, death occurred on the thirteenth day. The temperature was high, reaching 39.7° C. Pus from the ear and nares, and the mucous secretions from the larynx and trachea revealed only the pyocyaneus bacillus. In this contribution Kossel states that in the investigation of 52 cases he has found the pyocyaneus bacillus eight times, usually in association with other bacteria. Three times it was present in the blood and in the exudates from the middle ears of children. Krannhals<sup>102</sup> records the case of a man who died 27 days after an operation for empyema. This infection took place in a powerful adult. Pus from the mediastinum, pericardial exudate, and material from the spleen showed only the pyocyaneus bacillus in culture. In a second case with similar symptoms only the pyocyaneus bacillus was grown from the intestinal lesions. No typhoid bacilli were present. Kuehn<sup>103</sup> obtained the pyocyaneus bacillus in pure culture in a case of septicemia from the spleen one hour after death. It was also isolated from other organs. Lannay<sup>104</sup> in an exhaustive contribution divides pyocyaneus infections into local and general, the latter being severe septico-pyemias, which nearly always terminate fatally. He affirms that general pyocyaneus infections frequently originate from primary intestinal lesions such as enteritis, dysentery, and typhoid fever. The bacillus is found in the feces, blood, mesenteric glands, and ecchymoses of the mucosa of the intestine. Lannay says he has often isolated this organism from many springs at well-known watering places. Manicatide<sup>105</sup> found this germ in one case in pure culture post mortem in a child

14 months old in the spleen, kidney, and heart blood. In all the other tissues and organs investigated it was found, but not in pure culture. In a second case, in a four-year-old child, a septicopyemia developed following an attack of diphtheria. The pyocyaneus bacillus was present in pure culture in the spleen, kidney, and heart blood. Neumann<sup>106</sup> records three cases in which *B. pyocyaneus* was present. The first occurred in an infant 13 days old. Death terminated the illness which was characterized by the presence of icterus, petechiae, and hemorrhages of the mucous membranes. The pyocyaneus bacillus was grown from the blood and organs post mortem in pure culture. In another contribution he describes two cases of hemorrhagic diathesis in the new-born. In one of these there was a typical melena. In the other, which occurred in an infant suffering from congenital syphilis, there was a hemorrhagic diathesis. From both cases many colonies of the pyocyaneus bacillus were grown from the pleural and peritoneal fluid, from the intestinal contents, and from the liver and spleen. There were also a few coccus colonies present. In the first of these two cases the *B. aerogenes* was obtained from cultures of the spleen and blood. Oettinger<sup>107</sup> reports the case of an 18-year-old male who, on the twentieth day of an illness, which had been diagnosed typhoid fever, suffered a relapse. On the twenty-fourth day a sudden rise in temperature occurred, accompanied by a vesicular eruption with hemorrhagic contents. The vesicles were present in the greatest numbers on the scrotum, in the groin, and in the lumbar region. From the vesicles the *B. pyocyaneus* was grown in pure culture. Perkins<sup>108</sup> describes a case in a colored infant four months old. After an illness of only a week, during which there were numerous green dejections, the patient succumbed. No cultures were made from the intestines nor from the intestinal contents. In all the organs the pyocyaneus bacillus was present in small numbers in pure culture. In another case it was obtained from the lung in pure culture. There were also miliary abscesses in the brain but no cultures were made from them. In a third case following a burn of the third degree the pyocyaneus bacillus was present in the heart blood in small numbers. Rolly<sup>109</sup> reports a case of general septicemia in a young woman who had aborted. She suddenly showed signs of sepsis, meningitis, etc. On the fourth day of her illness the pyocyaneus bacillus was obtained

in pure culture from her blood and again on the eighth and tenth days of her disease. On the eleventh day she died and the same organism was obtained from the heart blood, kidney, spleen, intestines, and other organs. The point of entrance was apparently the puerperal uterus. Triboulet and Tollemer<sup>110</sup> report a case of septicemia in a child of eight months. It apparently took its origin from disseminated round and cup-shaped ulcers of the skin from which the pyocyaneus bacillus was isolated. Post mortem the germ was found in the heart blood. Sections of the skin showed only a superficial infection, the capillaries not being invaded. Apparently the septicemia had arisen from the skin lesions. Williams and Cameron<sup>111</sup> found the *B. pyocyaneus* in pure culture in two cases in children with clinical symptoms of septicemia. In one it was obtained from the spleen and kidney but not from the heart blood. In the other it was found in pure culture in the liver, spleen, and kidney. The heart blood and urine were sterile. In two other instances it was encountered by them, once in a subcutaneous abscess in association with the *Staph. citreus*, and once in the cecum.

#### MISCELLANEOUS INFECTIONS.

Babes<sup>112</sup> obtained the *B. pyocyaneus* in pure culture post mortem from abscesses which had their origin in septic umbilical veins. Boynton<sup>113</sup> records a case of infection due to the pyocyaneus bacillus in which the primary focus was in decubitus. Charrin<sup>114</sup> isolated it in pure culture from the greenish discharge of a chronic mastitis and from a tumor of the leg in a young man. The tumor resembled a gumma but there was no evidence of either syphilis or tuberculosis. Curry<sup>115</sup> in the bacteriological examination of six cases of pustule formation found it in pure culture once; in six cases of furunculosis once; in 35 cases of cellulitis three times. Eisenberg<sup>116</sup> reports the case of a man 43 years old, who, four months previous to the time he was first seen, had injured the right leg close to the knee joint. A tumor formed which was very painful. Death occurred at the end of five months. The purulent wound secretions were examined bacteriologically for the first time nine days before death. Plate cultures showed a white coccus and the pyocyaneus bacillus; four days before death a white coccus, pseudo-diphtheria bacilli, and the

pyocyaneus bacillus. Jadkewitsch<sup>117</sup> observed a patient who, in the course of 10 years, was attacked with ulcers of the leg three times which were in all probability caused by the pyocyaneus bacillus. In the first attack the blue pus lasted for three months. Five years later it again appeared and lasted three weeks. The third attack appeared three years later and lasted with intermissions for four months. Clinically there was some cutaneous anaesthesia and motor paralysis. The pyocyaneus bacillus was grown from the urine. Lanz and Luescher<sup>118</sup> isolated from the pus, in a case of purulent strumitis, which was incised one month after the beginning of the disease, the pyocyaneus bacillus. Wassermann<sup>119</sup> contributes 11 cases of umbilical infection, four of which were examined bacteriologically. All those examined contained *B. pyocyaneus* in pure culture. In seven of these cases metastases occurred with foci of septic pneumonia in which there was hemorrhagic, cellular, and fibrinous exudation, necrosis, and abscess formation. Inoculations in animals with pure cultures of this germ produced similar lesions.

#### INFECTIONS OF THE LIVER.

Kruse and Pasquale<sup>120</sup> in the examination of nine cases of liver abscess, in which there was no history or evidence of a dysenteric origin, isolated it three times. Once it was present in pure culture; in the other two cases the cultures were almost pure. In the examination of six more cases complicating dysentery it was never present. The first case was that of a Greek who had lived in Egypt 12 years. Five years previous and again three months before his admission to the hospital he had suffered from a temporary diarrhea. For 20 days he had suffered from pain in the right hypochondrium and in the right shoulder, chills, and fever. Venereal and alcoholic excesses were denied. From the increased liver dulness a diagnosis of abscess of the liver was made. On the same day the liver was punctured. There was little pus present, the blood and pus corpuscles being well preserved. There were no amebae present. Countless colonies of *B. pyocyaneus* were isolated in pure culture.

The second case occurred in a Greek who had always lived in Egypt. He had never suffered from dysentery or diarrhea. Three years before and again one year previous to his admission to the

hospital he had had attacks of pain in the liver region but there was no evidence of fever. Two months before he appeared at the hospital the pain again returned, accompanied by chills and fever. The diagnosis of hepatic abscess was made. The abdomen was opened by a longitudinal incision in the right parasternal line under the border of the rib. From the pus, which was very abundant, no amebae could be obtained. On agar plates there was an abundant development of *B. pyocyaneus* with a few colonies of a large motile bacillus which liquefied gelatin and produced gas. Healing of the abscess and recovery followed.

In the third case many colonies of the pyocyaneus bacillus were grown from cultures made from pus taken from the liver and smaller numbers from the peritoneum, spleen, and kidney. There were also present a few colonies of *B. clavatus* and typhoid-like organisms.

In the case of multiple liver abscesses reported below it will be seen that the pain in the right hypochondrium was much less severe and its site was less definitely located than in those of Kruse and Pasquale. The evidence of severe intoxication appears to have been less marked in their cases. From their description one would infer that there was a single focus of infection instead of multiple foci. Diarrhea was common to all but apparently less severe in the cases which they describe.

*Case I.*—K. M. American by birth, seven years old. The patient was first seen by Doctor H. J. Lehnhoff on September 7, 1907. She complained of a slight pain in the right side, loss of appetite, and of being hot.

*Family History.* Three brothers of the patient's father died relatively early—one at the age of 36 of Addison's disease; another at 32 of pulmonary tuberculosis; a third at 32 of the same disease. The history was otherwise negative.

*Previous History.* The patient had had two attacks of bronchopneumonia, the first occurring at the age of three and the second in May, 1907. From both she apparently recovered in the course of a few days. Except for pain in the epigastric region, of which she complained rather frequently during the last year of her life, she had always been vigorous and seemed to be well in every respect.

*Present Disease.* On the morning of September 7, 1907, the

patient was seen for the first time. Examination revealed nothing except slight tenderness of the abdomen in a circumscribed spot corresponding to McBurney's point. There had been no chill, no abnormality of bowel movements, no vomiting, and very little pain. A tentative diagnosis of catarrhal appendicitis was made. The child was put to bed and an ice bag placed over the tender spot. On the evening of the same day the patient was seen again and found to be in practically the same condition as in the morning. The next day the child seemed to be better and her parents were instructed to let her get up the following day if she continued to do well.

During the next few days she seemed well again and played out of doors as usual. On the twelfth she complained of the same pain again. At this time she seemed to be a little more depressed; the pain was located higher in the abdomen but the area of tenderness could not be definitely located. Her tongue was coated; there was a slight elevation of temperature; and she had little desire for food. No diagnosis was made at this time. Since the symptoms, though rather indefinite, suggested the possibility of typhoid fever, the agglutination test was tried. This proved to be absolutely negative.

A day or two later the child had a chill which lasted for 20 minutes followed by a rise in temperature. From this time on there was a chill almost daily, sometimes two in 24 hours. The temperature rose to a higher point each day until it reached  $104^{\circ}$  F. One evening the temperature was reported by her parents to be  $106^{\circ}$  F. On visiting her an hour and a half later it was found to be considerably lower and it never at any subsequent time reached this point. The stools became semi-fluid, were of a reddish-brown color, and the dried fluid portion left a saffron-tinted stain. There was movement of the bowels at least once every day, sometimes twice, without any artificial aid. At this time the patient complained very little of pain and did not seem very ill. During the intervals between her febrile attacks she would get out of bed and walk about the room. About the fifteenth of September it was first noticed that the veins on the right side of the abdomen were somewhat enlarged, being much more clearly visible than those of the left side. There was apparently a mass which could be detected by palpation at the lower border of the ribs on the right side.



Doctor A. R. Mitchell was called in consultation and a diagnosis of acute hepatitis of unknown origin was made. Since the symptoms indicated the probability of pus somewhere and from the fact that some enlargement of the liver could apparently be detected an exploratory incision was advised. This was made on September 20. The appendix was removed since it was found to be enlarged and congested. The liver appeared to be somewhat enlarged and was of a darker color than normal on account of congestion. There was no visible evidence of abscess, nor could any fluctuation be elicited. The gall bladder appeared to be normal.

The patient recovered from the operation but the chills still recurred once or twice daily. There were one or two bowel movements each day, the color changing from a brownish tint to a green. The child now began to vomit and was finally unable to retain either food or water. The chills were frequently followed by a marked collapse. The vomitus and the feces had a very offensive odor. The patient gradually grew worse and died on October 3. On the evening of the same day a partial autopsy was held.

*Post-mortem findings.*—Unfortunately a complete post-mortem examination was not allowed. The abdomen alone was opened, but there seemed to be very little evidence of anything which would account for the clinical symptoms except the liver. This was found to be considerably enlarged. The surface was smooth and dark colored, and showed marked congestion. There was no evidence of abscess formation on the surface but on section numerous multiple abscesses varying in size from less than a millimeter to one centimeter in diameter were revealed. Cultures were made from the pus on several tubes of inclined plain and glycerin agar and from these the *B. pyocyaneus* was obtained in pure culture. Microscopical examination of stained smears from the pus and from the necrotic walls of the abscesses showed only small bacilli and these were present in enormous numbers. Sections taken from the liver substance also showed many organisms of similar appearance. These appeared to be identical with those obtained from the cultures.

In order to determine the pathogenic effects of this organism inoculations of broth cultures were made in white rats, guinea-pigs, and a rabbit. Three adult white rats received respectively through

intraperitoneal injection 1.0, 0.5, and 0.25 c.c. of a 24-hour broth culture grown at 37° C. All the animals died within 48 hours. From the heart blood, spleen, liver, and kidney of each *B. pyocyaneus* was obtained in pure culture.

Four guinea-pigs were inoculated intraperitoneally with broth cultures grown at 37° C. for 24 hours. The first received 1.0 c.c.; the second and third 0.5 c.c.; the fourth 0.25 c.c. All died within 48 hours and cultures made from the heart blood, spleen, kidney, and liver showed only *B. pyocyaneus*.

One large white rabbit received an intraperitoneal injection of 0.5 c.c. of a broth culture of the same organism grown for 24 hours at 37° C. This animal died on the third day, *B. pyocyaneus* being obtained in pure culture from the heart blood.

Guinea-pig No. 5 was immunized in the following manner. On November 21 it received an intraperitoneal inoculation of 1.0 c.c. of a broth culture heated for seven minutes at a temperature of 65° C. Four days later it received 1.0 c.c. of a broth culture heated for five minutes at 50° C. After another interval of four days it received 0.5 c.c. of an unheated broth culture. Five days later it received 1.0 c.c. of an unheated culture grown at 37° C. for 24 hours.

On December 5 guinea-pig No. 5 and a control guinea-pig No. 6 each received 1.0 c.c. of a broth culture of the same organism grown for 48 hours at 37° C. At intervals of from 5 to 10 minutes material was withdrawn from the peritoneal cavity of each guinea-pig and immediately examined in hanging drop. At the end of five minutes material removed from the peritoneal cavity of guinea-pig No. 5 showed some agglutination. At the end of 10 minutes there was some evidence of bacteriolysis and at the end of 30 minutes nearly every organism had undergone solution and disappeared. Guinea-pig No. 6 at the end of 30 minutes showed bacilli in apparently as large numbers and as actively motile as in the beginning of the experiment. There was evidence neither of agglutination nor bacteriolysis.

Culturally the organism, isolated from this multiple infection of the liver, resembled closely *B. pyocyaneus* ( $\beta$ ) described by P. Ernst. The resemblance was so striking that a description was deemed

unnecessary since it would add nothing of value to the literature of the cultural and morphological characteristics of *B. pyocyaneus*.

#### INFECTIONS OF BONES AND JOINTS.

Pawlowsky<sup>121</sup> isolated *B. pyocyaneus* from a tuberculous joint. Perkins<sup>122</sup> found it in pure culture in one case in abscesses in the region of the seventh rib on the left side and in the left elbow joint. Schuermayer<sup>123</sup> in a prepatellar bursitis, in which there was a serous exudate, obtained from the fluid a pure culture of the pyocyaneus bacillus which he placed between ( $\alpha$ ) and ( $\beta$ ). His case shows that this germ may cause an increased secretion without any pus formation.

The following is the history of another infection of a joint which was obtained through the courtesy of Drs. O. C. Reynolds and E. W. Rowe.

*Case 2.*—The patient was a young woman about 20 years of age, a Scandinavian by birth. She was married and had given birth to one child. Delivery had apparently been normal and nothing unusual had occurred following parturition. Nothing of importance in her previous history could be discovered except the unhygienic surroundings of her home.

Early in March, 1903, she was taken suddenly ill. There was intense pain and much swelling of the knee joint. The pain was so intense that morphine in large doses gave only partial relief. All the symptoms of an acute osteo-myelitis were present. Fever from the first was high and ushered in with a chill. About 10 days after the onset she was taken to the hospital and a portion of the head of the tibia resected. On opening the joint a large quantity of pus was found. The anterior portion of the articular surface, and the anterior border of the head of the tibia, and the posterior surface of the patella were necrotic, the necrosed portions looking as if they had been gnawed by a rat. At the time of the operation the patient was in a very poor condition. The knee was placed in a plaster cast and kept there for about two weeks. At the end of this time it was decided that an amputation was necessary, since the pain was intense and the patient was rapidly getting worse. The pus had a very offensive odor and when the cast was removed it was found that there was very little evidence of any change in the condition of the lesion except

more extensive necrosis of the bones and soft parts. It was therefore thought best to do a mid-thigh amputation. After the operation the stump healed very rapidly and the patient quickly regained her strength. In about two weeks she was discharged with only a slight opening in the stump which had not entirely healed.

From the pus obtained from the joint *B. pyocyaneus* was isolated in pure culture. A microscopical examination of stained spreads of the pus showed only bacilli which were the same in appearance as those grown on culture media. This organism corresponded in almost every detail with the *B. pyocyaneus* ( $\beta$ ) of P. Ernst. It is to be regretted that inoculations of this organism were not made in animals to determine its virulence.

#### SUMMARY.

After reviewing pyocyaneus infections one may perhaps be justified in drawing the following conclusions:

1. That primary infections may be caused by *B. pyocyaneus*.
2. That pyocyaneus infections are not always mild but may be of such severity as to cause death.
3. That lesions of the skin caused by this organism are at times only local manifestations of a general infection.
4. That some infections with symptoms resembling those of typhoid fever, which do not respond to the agglutination reaction for either typhoid or para-typhoid bacilli, may be due to the *B. pyocyaneus*.
5. That the middle ear and intestines are the most frequent foci of infection.
6. That a general septicemia may arise from any primary focus but most frequently arises from an infection of the intestinal tract.
7. That *B. pyocyaneus* may be the specific cause of both sporadic and epidemic diarrheas and dysenteries.

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## A METHOD FOR THE STAINING OF NEGRI BODIES.\*†

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SINCE the description by Negri in 1903 of certain bodies found in the brains of animals dying of rabies, many methods have been recommended for the staining of these structures. This is in itself evidence that all of these methods are more or less unsatisfactory at times. Although in most cases the Negri bodies are numerous, large, and



FIG. 1.—Photo-micrograph from cornu ammonis of a dog made three days after death. (Post mortem bacterial invasion.) Negri bodies lying within nerve cells.  $\times 1,000$ .

easily recognized, investigators are agreed that in some cases this recognition is very difficult and uncertain.

During the past 18 months dogs suspected of being rabid, including those killed by the police, have been sent to the laboratory of the city bacteriologist of St. Louis, for diagnosis. In the beginning of this work we were occasionally unable to demonstrate the presence of Negri bodies in animals which were proven to be rabid through inoculation tests. In the past year we have developed an eosin methylene blue stain which has proven so satisfactory and is so rapid and simple that it seems desirable to report the method.

\* Received for publication October 29, 1908.

† Read before the Laboratory Section of the American Public Health Association, Winnipeg, Canada, August 26, 1908



In the routine examination smears between two glass slides are made from the cornu ammonis, cerebellum, and cerebral cortex. Two slides are preferable to one slide and one cover glass, as the thickness of the smear can be controlled more easily and there is less danger of soiling the hands. The smear is to be treated as follows:

Fix in methyl alcohol one minute.

Wash briefly in water to remove the alcohol.

Immerse in an old saturated solution (95 per cent alcohol) of alcohol soluble eosin from one to three minutes.

Wash two or three seconds in water to remove the excess of eosin.

Immerse in a fresh solution of Unna's alkaline methylene blue 5 to 15 seconds.

Wash briefly in water.

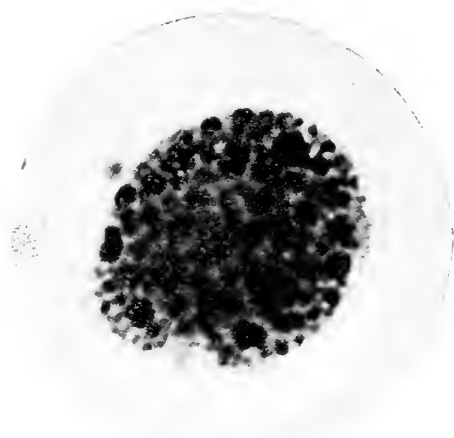


FIG. 2.—Photo-micrograph of Negri body in a smear from the cornu ammonis of a dog.  $\times 3,000$ .

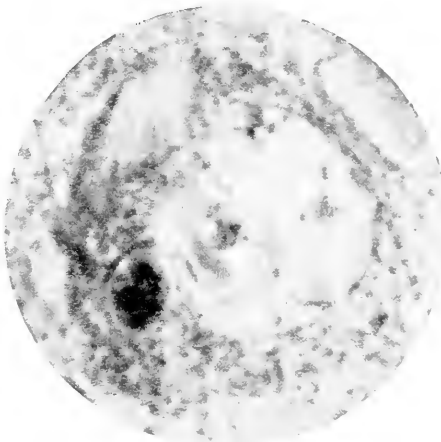


FIG. 3.—Photo-micrograph of a section from the cornu ammonis of dog, showing a Negri body lying within the nerve cell.  $\times 3,000$ .

Decolorize in 95 per cent alcohol; and follow with absolute alcohol, xylol, and balsam, or blot and dry in the air.

The entire process requires less than five minutes.

The structure of the bodies is more sharply defined if the smear is not allowed to dry before being fixed and stained. Smears which have dried for several days or weeks cannot be stained satisfactorily.

The older the saturated solution of eosin (alcoholic), the more rapidly and intensely it stains. A solution less than two months old will not yield good results.

The methylene blue (Unna's) will produce a very disturbing precipitate if it be older than a week or two. The fresher the solution, the more sharply defined are the "inner" bodies.

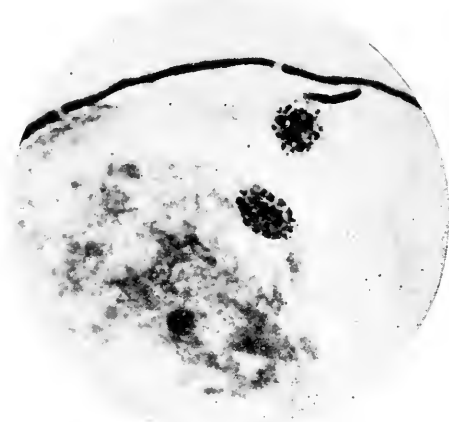


FIG. 4.—Photo-micrograph from cornu ammonis of a dog made three days after death. (Post-mortem bacterial invasion.) Negri bodies lying within nerve cells.  $\times 1,000$ .

During the decolorization the slides should be examined from time to time. Decolorize until only the nerve cells are blue and the

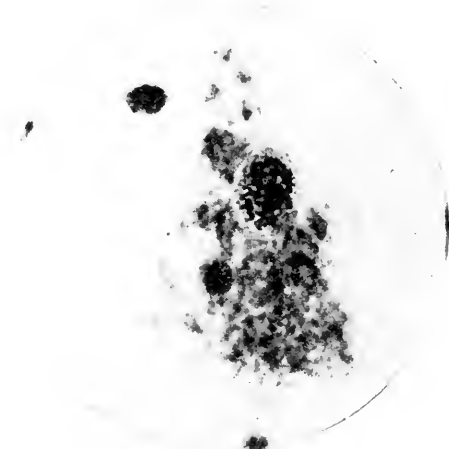
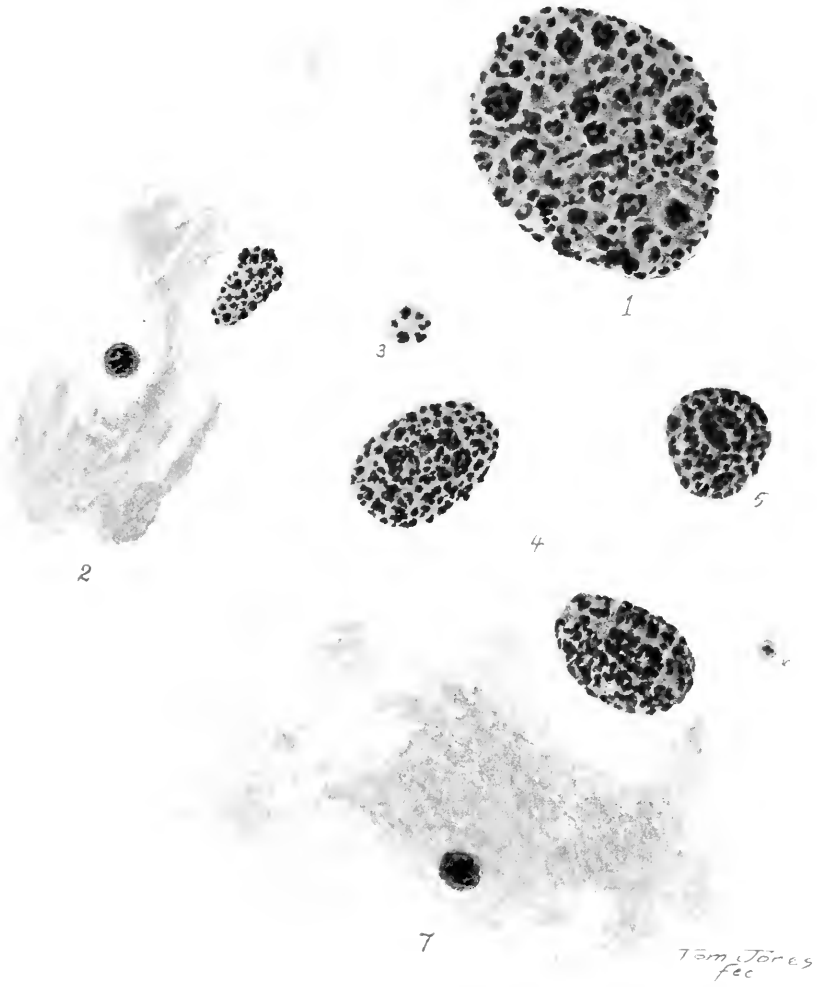


FIG. 5.—Photo-micrograph from cornu ammonis of a dog made three days after death. (Post-mortem bacterial invasion.) Negri bodies lying within nerve cells.  $\times 1,000$ .

red cells have become bright red. It has been our experience that when the red blood cells appear red, the nucleoli of the nerve cells a

PLATE 5.





deep blue, and the protoplasm a pale blue, the Negri bodies will be seen colored a light red with sharply defined dark bluish-red "inner" bodies. It is better to decolorize too much than too little. The results will be more uniform if the staining is done in staining-dishes or jars.

The advantages of this method are:

Strong contrast between Negri bodies, nerve cells, and blood cells; the absence of a granular precipitate, so disturbing in many methods

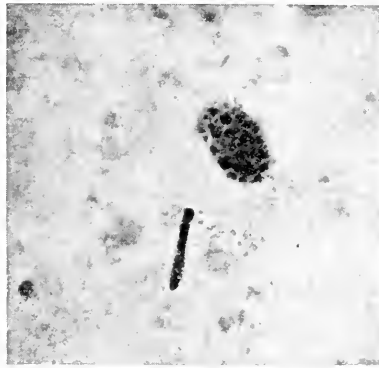


FIG. 6.—Photo-micrograph of Negri body from cornu ammonis of dog. Smear.  $\times 1,000$ .

as to render the recognition of the small-sized bodies impossible; rapidity and simplicity.

In the staining of material fixed in Zenker's fluid or acetone, the sections should be stained three to five minutes in the eosin and 30 seconds to two minutes in the methylene blue. In sections the inner bodies appear a deep blue.

A little practice and familiarity with the solutions employed will enable one to judge correctly the time necessary for each step.

#### DESCRIPTION OF PLATE 5.

Drawings showing the Negri bodies in smears from the cornu ammonis of a dog. (Bacteria omitted.)

Fig. 1 corresponds to photo-micrograph 2.  $\times 3,000$ .

Fig. 2 corresponds to photo-micrograph 1.  $\times 1,000$ .

Figs. 5 and 7 correspond to photo-micrograph 4.  $\times 3,000$ .

# ON THE OCCURRENCE OF THERMOSTABLE AND SIMPLE BACTERICIDAL AND OPSONIC SUBSTANCES.\*

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IN an article entitled "Further Studies on Virulent Pseudo-diphtheria Bacilli" published two years ago,<sup>1</sup> Dr. Horton and I described the properties of the serum of animals immunized against the "Ruediger bacillus," an organism belonging probably to the pseudo-diphtheria group. The serum of rabbits and of goats which had been immunized against the bacillus presented certain peculiarities. It contained specific agglutinin, opsonin, and bacteriolysin for these bacilli and showed itself extraordinarily resistant to heat, age, light, and drying. After the serum had been partially or wholly inactivated by heat, it was found to be impossible to reactivate it by adding fresh complement. Experiments in removing amboceptor by absorption also failed, and the bactericidal substance apparently did not consist of complement and amboceptor. The opsonin was also found to be highly resistant to heat and attempts to reactivate heated serum failed, but opsonin and bacteriolysin were apparently not the same substance.

The conclusions drawn at the end of the work were as follows: "The study of the bacteriolysin and opsonin of our immune serum reveals the fact that we have here an immune serum of high degree of thermostability, apparently not containing complement and amboceptor.

"Experiments on guinea-pigs indicate that the immune serum causes a marked polynucleosis and increases phagocytosis *in vivo* as well as *in vitro*, hence the protective action of this serum must be ascribed in part at least to the immune opsonin.

"The bacteriolysin and opsonin of these immune sera are not the same substance."

\* Received for publication October 31, 1908.

<sup>1</sup> *Jour. Infect. Dis.*, 1906, 3, p. 128.

The advances made in the last two years in our knowledge of antibodies, especially the opsonins, made it seem advisable to review and extend the work on this serum with regard to the two disputable points; namely, that the bacteriolysin and opsonin do not contain complement or amboceptor, and second, that the thermostable bacteriolysin is not identical with the thermostable opsonin.

There are a number of recent publications dealing with heat-resistant antibodies, hemolytic and bactericidal, some being even "coctostable," resisting the temperature of boiling water. Thus Korschun and Morgenroth,<sup>1</sup> Woelfel,<sup>2</sup> Levaditi,<sup>3</sup> and Conradi<sup>4</sup> have all described heat-resistant lytic substances extracted from animal cells. These bodies are not complex, i. e., not resolvable into an amboceptor-complement group. Other thermostable antibodies, both hemolysins and bacteriolysins, have been shown to consist of complement and amboceptor. Such are the endolysins of Schattenfroh<sup>5</sup> and Pettersson,<sup>6</sup> thermostable bodies extracted from disintegrated leucocytes. A bactericidal blood serum that owes this property to a substance which is thermostable and not complex has not been described so far, and it is with some diffidence that I claim these properties for the serum studied in this paper.

#### NATURE OF THE BACTERICIDAL SUBSTANCE.

The serum of rabbits is normally bactericidal for the "Ruediger bacillus," although not strongly so. Plates made from tubes containing one part of normal serum to four of broth usually show a diminution of one-half to one-fourth of the number of colonies, when compared with the plates from control tubes. The bacteriolysin increases greatly after immunization, reaching in some instances 80 times its original strength. This increase is not, however, steady and continuous, but occurs in waves, the low-water mark falling to normal, or lower, and this even after repeated injections. Typically there is a rise at each injection followed by a fall and occasionally preceded by one, i. e., a negative phase. The curve in Chart 1 shows the variations in the bactericidal substance from day to day, and serves

<sup>1</sup> *Berl. klin. Wchnschr.*, 1902, 39, p. 870.

<sup>4</sup> *Beitr. z. chem. Physiol. u. Pathol.*, 1901, 1, p. 516.

<sup>2</sup> *Jour. Infect. Dis.*, 1905, 2, p. 97.

<sup>5</sup> *Munch. med. Wchnschr.*, 1898, 45, p. 1109.

<sup>3</sup> *Ann. de l'Inst. Pasteur*, 1903, 17, p. 187.

<sup>6</sup> *Centralbl. f. Bakt.*, 1907, 45, p. 237.

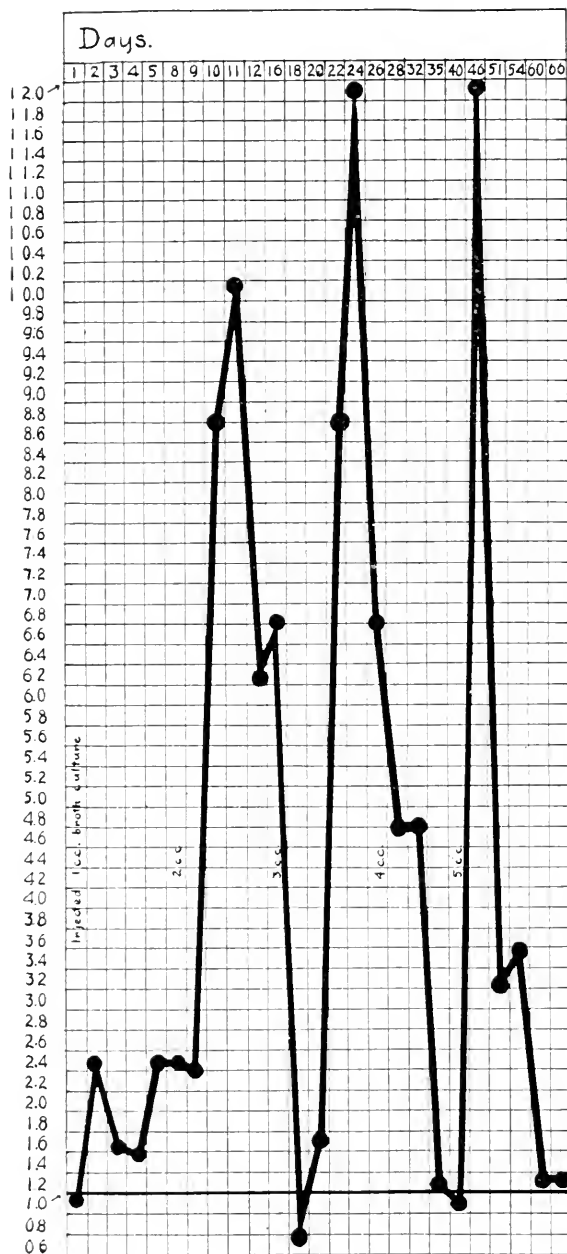


CHART I.—Curve showing fluctuations of bactericidal substance in the blood of a rabbit during immunization.



to explain why, in our former work, we sometimes obtained sera of very low bactericidal power from animals supposedly highly immune.

The amount of bacteriolysin present was estimated by comparing the plates made from tubes containing immune serum with the plates made from similar tubes containing serum from two normal rabbits. Thus, if these control plates contained 500 colonies, and the plates from the tubes with immune serum contained only 50 colonies, the bactericidal power of the immune serum would be 10.

Goat serum differs from rabbit serum in that it is normally strongly bactericidal to this bacillus, and immunization has practically no effect in increasing the amount of bacteriolysin. Three goats were injected with the same quantities of broth culture of the Ruediger bacillus, and in all the result was the same, a very slow and insignificant increase in the bactericidal substance.

The bactericidal substance is stable, not only in immune serum, but also in normal serum. Normal rabbit serum loses its bactericidal power only after one week's exposure to daylight, at room temperature, and normal goat serum can endure 12 days. Both can be heated to 70° for 10 minutes without complete inactivation. The immune serum of both animals is still more resistant to heat and age. Immune rabbit serum is not inactivated by exposure to direct daylight for a period shorter than 15 days. When kept in the ice-box, immune goat serum has retained its bactericidal power for more than four months, provided it is left in contact with the clot, deterioration taking place more rapidly if the serum is removed from the clot. Immune rabbit serum dried to powder and redissolved in broth is highly bactericidal. As to the effect of heat, immune serum resists long exposure to high temperature, the difference between normal and immune serum being shown not so much in the degree of heat which can be borne but in the period of time which the exposure can be prolonged without complete inactivation. Thus normal serum can be heated to 70° C. for 10 minutes without complete inactivation, and immune serum is inactivated by exposure for the same length of time to 80°, but normal serum is destroyed by two hours' exposure to 55°, while immune rabbit serum can survive 24 hours at this temperature and immune goat serum, three days. The increase in the bactericidal substance of the goat serum after immunization is thus made plainly

evident by applying the heat test. For, while the unheated serum may show no increase in bactericidal power over unheated normal serum, yet the latter will be completely inactivated by two hours at 55° while the former will have lost very little of its activity. The loss in bacteriolysin by heat is progressive, and the difference in heat resistance between goat and rabbit serum—the serum of the goat retains its power longer than that of the rabbit—is probably only a quantitative one.

It will be seen, then, that we have here a normal lysin of a high degree of thermostability, and that this lysin or lytic property of the serum increases under immunization. According to Dean, this should be explained by the fact that while the normal serum contains both complement and amboceptor the latter is present in small quantities only, and therefore normal serum is quickly inactivated. In immune serum, on the other hand, amboceptor is present in large quantities and heating, which destroys complement, causes only partial inactivation, because the amboceptor is capable of acting alone. The high degree of thermostability of our normal serum, however, argues against the presence of complement, and the experiments made in reactivation confirm the belief that the bactericidal substance in both immune and normal serum is simple and indivisible.

Various methods were used in attempts to determine the presence of complement and amboceptor. Exposure of a mixture of serum and bacilli to zero temperature resulted in the absorption of the bactericidal substance *in toto*. Bacilli which had been treated with immune serum at zero were removed, washed, and incubated, some with normal salt solution only, some with the addition of fresh normal rabbit serum. There was no difference between the two sets of plates except what could be accounted for by the bactericidal action of the normal serum.

In attempting to reactivate heated normal serum, the serum of sheep and human beings was chosen, as both are highly bactericidal to the Ruediger bacillus, and the serum of immune goats and rabbits was used for testing immune serum. The serum was heated to 64°–70° for 10 to 30 minutes. As complement, the substances employed by different observers were used. Fresh serum in the proportions used by Dean, fresh living leucocytes, according to the

method of Schneider,<sup>1</sup> and disintegrated leucocytes, according to Pettersson's method, were added to the inactivated serum. The following is the result obtained in a typical experiment with inactivated immune serum and fresh complementary serum.

TABLE 1.

Mixtures		Colonies after 4 Hrs. at 37° C
Broth.....	1.0	6,400
Normal rabbit serum.....	0.2 + Broth 0.8	2,450
Immune goat serum.....	" + " "	2,000
Rabbit serum 1 part.....	" + " "	2,240
Immune goat serum 70° 2 parts.....	" + " "	1,672
Rabbit serum 2 parts.....	" + " "	1,404
Immune goat serum 70° 2 parts.....	" + " "	1,100
Rabbit serum 2 parts.....	" + " "	600
Immune goat serum 70° 1 part.....	" + " "	
Rabbit serum 4 parts.....	" + " "	
Immune goat serum 70° 1 part.....	" + " "	
Unheated Immune goat serum.....	" + " "	

There is evidently no real reactivation here. The next to the last tube in the series contained the proportions of serum recommended by Dean, namely, one part of inactivated to four parts of fresh serum, and here the number of colonies fell to one-half of the average for the two sera used separately. This result was invariably obtained in these experiments, a greater bacteriolytic effect from the use of the two sera in these proportions than from any other combination. Nevertheless, this can hardly be accounted reactivation, for the two sera are present in proportions exactly the reverse of those usual in reactivation experiments, and the resulting combination is only half as strong as the original unheated serum. Probably we have here a summation of effects due to the combination of two sera, both of which have some bacteriolytic power.

Schneider has described, under the name of "Leukine," certain bactericidal substances, which are secreted by living leucocytes under the stimulus afforded by inactivated immune serum. His experiments were repeated with leucocytes from the dog, rabbit, and guinea-pig, and with immune serum of goat and rabbit inactivated by heat. In each instance the phagocytic power of the leucocytes was tested in order to make sure that they were uninjured and active, but the result of these experiments was always negative; it was not possible to reactivate the serum by this method.

<sup>1</sup> *Münch. med. Wchnschr.*, 1908, 55, p. 409.

According to Pettersson there are three substances involved in bactericidal immunity, namely, opsonin, serum bacteriolysin, which is thermolabile and complex, and finally endolysin, a complex but thermostable body contained in the bodies of leucocytes, and recoverable from these after they have been subjected to repeated freezing and thawing. In order to discover whether the thermostable bacteriolysin of our serum were derived from the leucocytes, blood was drawn from a normal sheep, from an immune goat, and from an immune rabbit. The blood was defibrinated by beating, the serum removed immediately, and the upper layer of corpuscles withdrawn and freed from serum by washing in normal salt solution. A portion of these corpuscles was heated to  $68^{\circ}\text{C}.$ , and another portion was frozen and thawed three times; part of this latter was then heated to  $68^{\circ}$ . The bactericidal effect of serum, corpuscles, and frozen corpuscles was compared both before and after heating to  $68^{\circ}$ . An attempt also was made to reactivate the heated serum by means of frozen leucocytes, and the effect of unheated serum plus frozen leucocytes was compared with the effect of unheated serum plus uninjured leucocytes. Without giving the details of these experiments it will be sufficient to say (a) that the extract from frozen leucocytes was usually more strongly bactericidal than the suspension of uninjured leucocytes, but never so strong as the serum; (b) that the leucocytic extract was not more heat resistant than the serum, but less so, probably a purely quantitative difference; (c) that mixtures of heated serum and frozen leucocytes showed merely a summation of effects, no reactivation, and that (d) the addition of leucocytes, either living or disintegrated, to the unheated serum produced a mixture more strongly bactericidal than the serum alone. This last fact is in accordance with the observation repeatedly made in the course of this work, that serum removed from the clot by defibrination was less strongly bactericidal than serum left in contact with the clot. Undoubtedly the bactericidal substance is in part derived from the bodies of leucocytes.

As a result of these experiments it seems justifiable to assert that the substance in normal and immune serum bactericidal to the Ruediger bacillus is non-complex, and cannot be resolved into complement and amboceptor.

The bactericidal substance was precipitated from human serum and from immune rabbit serum by the addition of twenty parts of 98 per cent alcohol. The precipitate and the filtrate were both evaporated to dryness in the incubator, were then redissolved in broth, and tested:

TABLE 2.

Mixtures		Colonies after 4 Hrs. at 73°
Control broth.....	1.0	2,200
Filtrate in broth.....	1.0	2,500
Precipitate in broth.....	1.0	640

## NATURE OF THE OPSONIN.

The normal serum of the rabbit and of the goat contains opsonin for the Ruediger bacillus, goat serum averaging an index of about 1.5 as compared with rabbit serum. Normal opsonin deteriorates quickly, disappearing after three or four days' exposure to room temperature. It practically is destroyed by 15 minutes' exposure to 70°, by 30 minutes' exposure to 60°, and two hours' exposure to 45° C. In goat serum the degree of resistance is a little higher than in rabbit serum, probably a quantitative difference. On immunization the amount of opsonin increases in the typical manner, describing the sort of curve which we expect to follow injections of immunizing doses. Goat serum does not respond as well as rabbit serum in the formation of opsonin. Chart 2 shows the opsonic index of a rabbit immunized against the Ruediger bacillus. Immune opsonin is resistant to heat and to drying, although not to the same degree as immune bacteriolysin. The latter is still active after four hours' exposure to 64°, but immune opsonin is destroyed by two hours' exposure to that temperature.

This is a point of difference, however, upon which one would hesitate to lay stress, for it is well known that the Wright method of determining opsonin does not show small quantities of the substance while even a small amount of bacteriolysin is easily demonstrable.

Dean and others, notably Chapin and Cowie,<sup>1</sup> have succeeded in restoring the opsonic power to heated serum by the addition of fresh normal serum. Experiments in restoring the opsonin to our inacti-

<sup>1</sup> *Jour. Med. Res.*, 1907, 17, pp. 37, 95, 213.

vated serum were as unsuccessful as the experiments in restoring bacteriolysin. The following is a typical experiment with heated immune goat serum and fresh normal rabbit serum:

TABLE 3.

Mixtures		Average No. of Bacilli per Leucocyte	
Rabbit serum.....		2.2	} 2.36
Goat serum heated to 70°.....		0.16	
1 part rabbit serum + 1 part goat serum 70°.....		3.75	
1 " " " 2 " " " ".....		2.25	
1 " " " 3 " " " ".....		2.8	
1 " " " 4 " " " ".....		1.3	
2 " " " 1 " " " ".....		3.75	
3 " " " 1 " " " ".....		2.8	
4 " " " 1 " " " ".....		4.3	

Table 3 shows that phagocytosis is greatest in the tube containing the largest quantity of unheated serum, and least in that containing

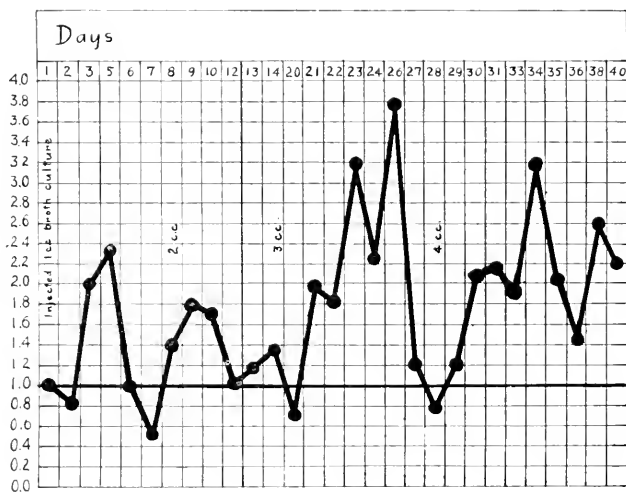


CHART 2.—Opsonic index of rabbit during immunization.

the smallest quantity. In the last tube we have almost twice as much phagocytosis as in the control, and this is in the tube containing the proportions recommended by Dean, namely, one part of amboceptor to four of complement; but here, as in the case of bactericidal experiments, one would hesitate to attribute the effect to reactivation.

Like the bacteriolysin, the opsonin is precipitated by the addition of 98 per cent alcohol.

TABLE 4.

Mixtures	Average per Leucocyte
Serum.....	5.8
Filtrate dried and dissolved in normal salt solution.....	0.4
Precipitate " " " " " " .....	4.0

The question as to whether the bactericidal and the opsonic properties of a given serum are due to the action of one body or of two distinct bodies is still unsettled. Dean maintains that they depend on one and the same substance, on the ground that sera which are strongly bacteriolytic for a certain organism are also strongly opsonic for that organism, both producing their effect through the combined action of a thermolabile complement and a thermostable amboceptor. The latter, Dean finds, is capable of acting alone after the complement has been destroyed by heat, but its effect is strengthened by the addition of fresh complement. This is true of both opsonin and bacteriolysin.

On the other hand, Neufeld and Hüne<sup>1</sup> argue against the identity of the two substances on the ground of a study of typhoid and paratyphoid sera. The serum of animals immunized against paratyphoid bacilli is not bactericidal to those organisms, but does contain specific opsonin for them. The serum of a typhoid patient may be bactericidal to typhoid bacilli and contain no opsonin at all, or but very little, while another, no more strongly bactericidal, may contain a decided amount of opsonin. Therefore, they conclude, in these two instances the opsonin and bacteriolysin are not identical.

Hektoen<sup>2</sup> in 1906 reviewed the literature previous to that date and, adding the result of his own researches, concluded that opsonins are distinct from bactericidal substances and from agglutinins for various reasons but more particularly because serum, normal as well as immune, may contain opsonin for a given organism, but not, at least as far as is yet known, the proper lytic amboceptor for that organism.

Pettersson also maintains the duality of these substances as does Schneider.

In the case of our immune serum there are several arguments in favor of the identity of the bactericidal substance and the opsonin. In the first place, both are heat resistant, both show an intermittent

<sup>1</sup> *Arch. a. d. kaiserl. Gesund.*, 1907, 25, p. 164.

<sup>2</sup> *Jour. Infect. Dis.*, 1906, 3, p. 434.

increase during immunization, both are apparently non-complex bodies, incapable of reactivation, both are held back by a fine filter and precipitated by alcohol. On the other hand, there are cogent arguments against their identity.

If the two substances were identical, then any serum which was rich or poor in one should be rich or poor in the other also, but this is not found to be the case. Human serum has an opsonic index only slightly above rabbit serum, but is far more strongly bactericidal, sometimes a hundred times more. Moreover, two specimens of human serum which have approximately the same opsonic index may differ in bactericidal power as much as 12 to 200.

The following table gives the opsonic and bactericidal indices of two human beings and of seven lower animals, the serum of rabbits being used as standard.

TABLE 5.

Serum	Bactericidal Index	Opsonic Index
Human No. 1.....	12.	1.3
Human No. 2.....	200.	1.2
Chicken.....	50.	1.2
Goat.....	20.	1.5
Rat.....	16.	5.0
Horse.....	12.	0.67
Sheep.....	7-10	0.5
Guinea-pig.....	2.5-3	1.0
Dog.....	2.0	0.3

The bactericidal content of the sera varies more than the opsonic, but it is plain that the two do not keep pace with each other, that a high opsonic index does not mean a high bactericidal index.

Again, though the bactericidal substance undergoes an intermittent, wavelike increase on immunization, as does the opsonin, yet the two curves do not correspond, but a low opsonic index may be coincident with a high bactericidal index and *vice versa*. Chart 3 shows the three curves, opsonic, bacteriolytic, and agglutinative, of the serum of a goat, and Chart 4 the same of the serum of a rabbit. These indicate that while the three antibodies are formed simultaneously they are not identical, for they do not vary at the same rate. The difference is shown even more strikingly when one studies the processes which occur in guinea-pigs injected with a lethal dose of the Ruediger bacillus and with a protective dose of immune serum.



Dr. Horton and I found that phagocytosis played a more or less important rôle in the protection of guinea-pigs against injections of this organism and that an increase of polymorphonuclear leucocytes occurs very quickly after an injection and is much greater in the

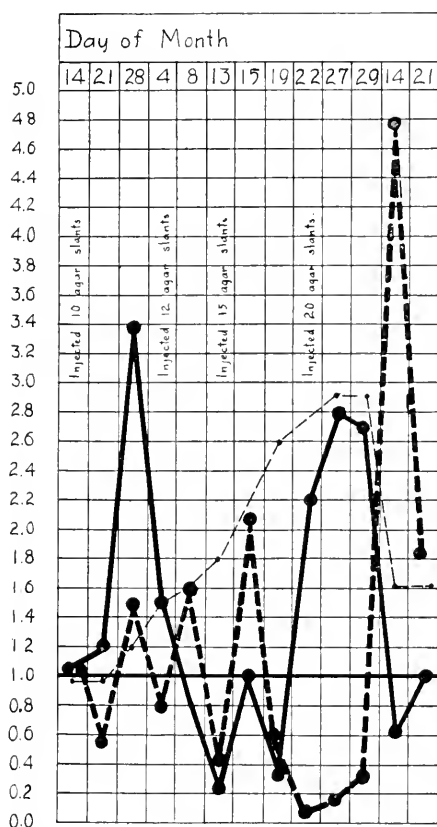


CHART 3.—Comparison of opsonin (solid line), bacteriolysin (heavy broken line), and agglutinin (fine broken line) in the blood of a goat during immunization.

guinea-pig which has received a protective dose of serum than in one which is not so protected. Two guinea-pigs were injected with lethal doses and at the end of three hours these two and a control animal were bled and a differential count of leucocytes made, with this result:

TABLE 6.

	Per cent Polynuclears
Control guinea-pig.....	1.6
Guinea-pig injected with broth culture Ruediger bacillus.....	8.6
Guinea-pig injected with broth culture Ruediger bacillus plus immune serum.....	44.0

It was also found that the peritoneal exudate of the protected guinea-pig contained a far larger number of polymorphonuclear leucocytes than did that of the unprotected animal. Guinea-pig A was killed three hours after receiving intraperitoneally 4 c.c. of a broth culture of B. No. 4. The exudate was almost clear with

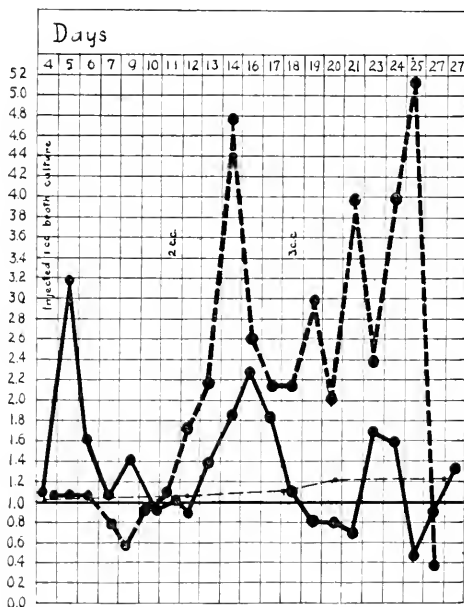


CHART 4.—Comparison of opsonin (solid line), bacteriolysin (heavy broken line), and agglutinin (fine broken line) in the blood of a rabbit during immunization.

many bacilli and but few leucocytes, 2 per cent of which were polynuclears, 94 per cent small mononuclears, and 4 per cent large mononuclears. Guinea-pig B received in addition to 4 c.c. of broth culture of B. No. 4, 2 c.c. of immune serum. It was killed at the end of three hours. The peritoneal exudate was slightly cloudy and contained a few agglutinated bacilli and many leucocytes, 54 per cent of which were polynuclears, 29 per cent small, and 12 per cent large mononuclears.

This increase in number of the polymorphonuclear leucocytes occurs early after the injection of culture plus protective serum. The withdrawal of small amounts of fluid from the peritoneal cavity of protected guinea pigs at short intervals after injection showed that phagocytosis of bacilli begins between one-half and one hour after the injection, reaches its height at the end of two and one-half hours, and is ended after six hours. The destruction of the bacilli is, however, much slower. Guinea-pigs which had been given a lethal dose of culture and a protective dose of immune rabbit serum were killed at different periods after the time of injection and cultures made from the blood and the organs. At the end of three hours, at the time when phagocytosis is at its height, a general invasion of bacilli has occurred and all body fluids and organs yield positive cultures. At the end of six hours cultures are obtained from the spleen and peritoneal cavity only, and at the end of 18 hours all cultures are sterile. Apparently then the opsonin is the protective substance first formed, the bactericidal substance later. Chart 5, which gives the curves of these antibodies in the blood of a guinea-pig after one injection of culture and immune serum, bears out this conclusion. The opsonin had increased at the end of three hours, during which time there was no increase of bacteriolysin, rather a slight fall. At the end of 20 hours the bacteriolysin was at its height and from then on it declined, while the opsonin, which was low at the end of 20 hours, rose again on the following day, as the bacteriolysin was falling.

It is possible that this bacteriolysin is of the nature of a toxin in Ehrlich's sense. In that case, it might be possible to produce an antitoxin by injections of immune serum. This has been attempted by injecting rabbits with immune goat and immune rabbit serum and guinea-pigs with immune goat serum, but the attempt thus far has proved a failure and there has been no production of antibacteriolysin.

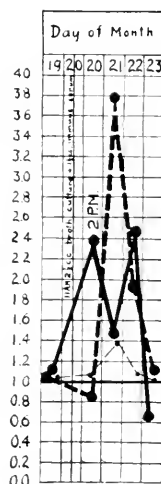


CHART 5.—Comparison of opsonin (solid line), bacteriolysin (heavy broken line), and agglutinin (fine broken line) in the blood of a guinea-pig following the injection of 2.5 c.c. of broth culture of the Ruediger bacillus and 1.5 c.c. of immune rabbit serum.

## SUMMARY.

In the blood of human beings and of some of the lower animals there is a thermostable bactericidal substance and thermostable opsonin for the Ruediger bacillus. These substances are increased on immunization and then show a much higher degree of thermostability.

The bacteriolysin of normal and of immune serum suffers a gradual loss on heating, but there is nothing to indicate that this is due to the loss of complement.

Experiments in reactivation of heated serum by means of fresh serum, of living leucocytes, and of disintegrated leucocytes show that the bacteriolysin and opsonin are not composed of complement and amboceptor.

The opsonin and bacteriolysin have many other properties in common, such as thermostability and precipitability by alcohol, but they are shown to be distinct by the fact that a serum rich in one of them may be poor in the other, and the further fact that while immunization causes an intermittent increase of both opsonin and bacteriolysin, the curves described by the two bodies are not parallel.

Experiments on guinea-pigs show that the first result of the injection of a broth culture of the Ruediger bacillus and a protective dose of immune serum is an increase of polymorphonuclear leucocytes in the circulating blood, the appearance of large numbers of phagocytes in the peritoneal cavity, and a rise in the opsonic index. At this time there is no demonstrable increase of bacteriolysin. Later on, the latter begins to increase and reaches its height after the opsonin has declined and phagocytosis has ceased. A second rise in the opsonic curve then occurs without any corresponding rise in the bacteriolysin.

It has not been possible to produce an antibacteriolytic serum by injections of immune serum in rabbits and guinea-pigs.

# EFFECTS OF INJECTIONS OF HOMOLOGOUS STREPTOCOCCI, KILLED BY HEAT, IN STREPTOCOCCUS COMPLICATIONS IN CONTAGIOUS DISEASES.\*

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IN view of the striking results reported to have followed the injection in persons suffering from various infections, of bacteria killed by heat according to the method advocated by Wright, it was thought desirable to test this method of treatment in patients with streptococcus complications in contagious diseases, especially in scarlatina.

We have seven such cases to report.

The bacteria injected were grown upon serum agar slants for 24 hours at 35° C. They were then suspended in 0.85 per cent sodium chloride solution and killed by heating to 60° for 30 to 45 minutes. In each instance cultures from the suspension after heating showed it to be sterile. The number of bacteria in the suspension was determined by Wright's method. The culture was always obtained from patient to be treated, from the purulent discharge from the abscess, the ear, or nose, or from the surface of the tonsil. Determinations of the opsonic index were made by Wright's method, the cultures from the individual being usually employed, although the indices closely corresponded, whatever streptococcus was used. The index was usually estimated daily and served as a guide to the injections. A pronounced fall in the index following the injection was rarely observed, but more or less rise was found after each injection except one. Slight local tenderness usually was found present at the place of injection for a day or two, and occasionally the local reaction was quite pronounced. Some idea of the character of the cases and their course can be gleaned from the following brief notes from the case records.

*Case I.*—Girl, age 5 years. Convalescent scarlatina. January 13, 1907: Profuse purulent discharge of four weeks' duration from a sinus following suppuration in the cervical lymph glands. 500,000,000 killed streptococci injected.

\* Received for publication November 10, 1908.

January 16: Discharge much less.

January 17: Discharge very slight. 500,000,000 killed streptococci injected.

January 20: Very little discharge.

January 21: 300,000,000 killed streptococci injected.

January 22: Developed chicken-pox. Discharge from sinus increased. Temperature 101° F.

January 28: 300,000,000 killed streptococci injected. Slight discharge continued for a month after the last injection during which time the opsonic index remained almost constantly above normal. (Chart 1.)

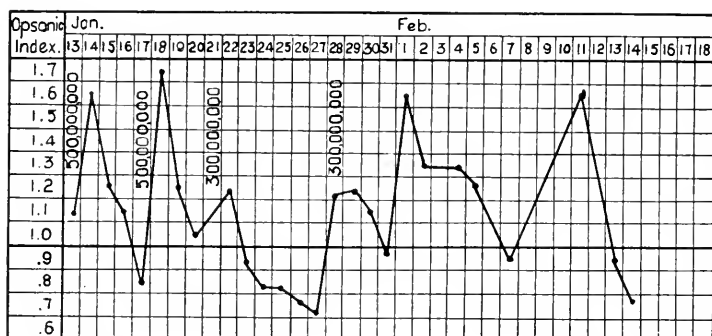


CHART 1.—Streptococco-opsonic index in Case I, with approximate numbers of bacteria injected.

*Case II.*—Boy, age 4 years. Cellulitis of the neck following diphtheria. January 15, 1907: Large, acutely swollen mass on the side of the neck was incised. No pus was found but cultures yielded abundant streptococci in pure culture.

January 17: A second incision opened a small pocket of pus.

January 19: 350,000,000 killed streptococci were injected.

January 25: 250,000,000 killed streptococci were injected.

January 30: Discharge almost ceased.

February 2: Discharged from hospital, the incision being almost healed. (Chart 2.)

*Case III.*—Girl, age 5 years. Scarlatina at end of first week, with profuse nasal discharge and much swelling of the cervical glands.

January 21, 1907: 250,000,000 killed streptococci injected.

January 22: Nasal discharge less; no change in glands.

January 25: 170,000,000 killed streptococci injected.

January 27: Nasal discharge much less.

January 28: 170,000,000 killed streptococci injected.

January 29: Nasal discharge stopped. Glands but little enlarged.

Uneventful recovery.

*Case IV.*—Girl, age 3 years. Scarletina at end of first week.

January 16, 1907: Glands on both sides of neck much swollen and hard. 600,000,000 killed streptococci injected.

January 19: Swellings incised; no pus found.

January 20: 300,000,000 killed streptococci injected.

January 22: Purulent discharge from incisions; general condition improved.

January 23: 250,000,000 killed streptococci injected.

January 26: Profuse hemorrhage from wound in neck.

January 27: Patient died.

Each injection was followed by a fall succeeded by a rise in the opsonic index.

*Case V.*—Woman, 20 years. Scarletina at end of first week.

January 21, 1907: Moderate enlargement of cervical lymph glands. 350,000,000 killed streptococci injected.

January 24: Glands more swollen and tender. 350,000,000 killed streptococci injected.

January 30: Glands much reduced in size. 350,000,000 killed streptococci injected. Uneventful recovery.

*Case VI.*—Boy, age 8 years. Scarletina, ten days after onset.

January 13, 1907: Fairly well-marked enlargement of the cervical lymph glands. 500,000,000 killed streptococci injected.

January 16: Glands much reduced in size and less tender and not so hard to the touch.

January 17: 500,000,000 killed streptococci injected.

January 22: Glands not tender and barely palpable.

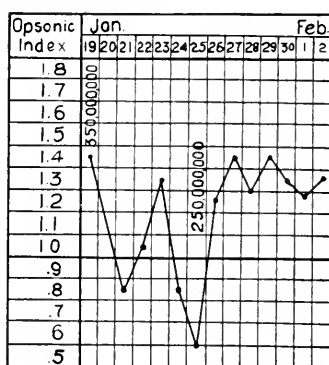


CHART 2.—Streptococco-opsonic index in Case II, with approximate number of bacteria injected.

In this case the glands were reduced in size much more rapidly than those in the case of the brother who received no injections. The conditions in the two patients before the injections were begun were very similar. (Chart 3.)

*Case VII.*—Man, 20 years. Suppurative otitis media, following scarlatina of a month previous.

January 31, 1907: 170,000,000 killed streptococci injected.

February 2: Discharge reduced about one-half.

February 5: 85,000,000 killed streptococci injected.

February 6: Aural discharge less.

February 8: Aural discharge stopped. (Chart 4.)

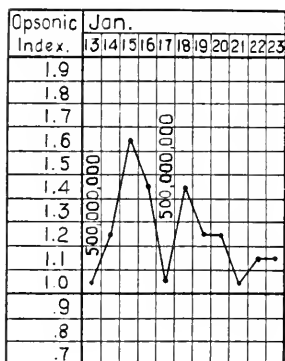


CHART 3.—Streptococco-opsonic index in Case VI, with approximate number of bacteria injected.

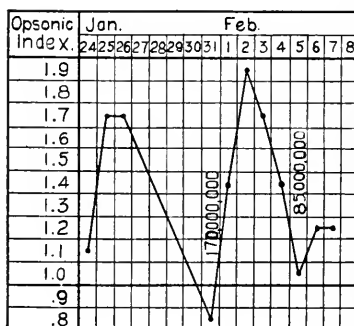


CHART 4.—Streptococco-opsonic index in Case VII, with approximate number of bacteria injected.

In watching these cases while under treatment it was very difficult to determine what effect, if any, upon the course of the disease could be ascribed to the injections. The impression, however, was obtained that in acute processes the effect was imperceptible. Because of the great variability in the natural course of these complications uninfluenced by treatment, it is almost impossible to judge the effects of treatment in individual cases. In some cases with enlarged glands which did not suppurate the recovery seemed to be accelerated. In arriving at our conclusions as to the effect of the injections upon the course of the infection, we have been guided by close observation of the cases, and by comparison with numerous uninjected cases. Dr. Baum, in whose service the cases occurred, and the resident physicians also formed the opinions expressed in our conclusions.



## A STUDY OF STREPTOCOCCUS IMMUNIZATION.\*

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### A STUDY OF THE IMMUNIZING EFFECTS AGAINST HOMOLOGOUS ORGANISMS OF INJECTIONS IN RABBITS OF STREPTOCOCCI KILLED BY HEAT AND KILLED BY CHEMICALLY INDIFFERENT AGENTS.

WHILE engaged in the study of streptococcus infections and immunity our attention was directed to some results obtained in connection with tubercle, typhoid, and glanders bacilli by Levy, Blumenthal, and Marxer.<sup>1</sup> They obtained a considerable degree of immunity in animals by the injection of bacteria which had been killed by chemically indifferent agents, such as sugars, glycerin, and urea. Virulent bacteria, devitalized by suspension in glycerin or strong solutions of sugars and urea, could be injected in relatively large quantities with little or no apparent effect upon the animal, but the injections were followed by a marked immunity for the corresponding living bacteria.

Having employed injections of streptococci killed by heat in the treatment of cases of streptococcus infection (see p. 585) without satisfactory results, we undertook to determine in animals the effects of the injection of virulent streptococci, killed by suspensions in a strong solution of galactose, and to compare the results thus obtained with those observed when streptococci killed by heat were injected. Rabbits were used in all the experiments, and the streptococcus cultures employed were made virulent for rabbits by passages through this animal. The streptococci were grown upon the surfaces of blood-agar slants in ordinary test-tubes. After 24 hours at 35° C. the fluid of condensation was removed and the bacteria upon the surface suspended in sterile, 25 per cent solution of galactose. Two c.c. of the solution was employed for each slant. The suspension was kept in the incubator for 48 or 72 hours, being shaken several times in the interval. The suspension was then distributed in small test-tubes,

\* Received for publication November 10, 1908.

<sup>1</sup> *Centralbl. f. Bakt.*, 1906, 42, p. 265.

each tube containing one-half the growth from a blood-agar slant. After thorough centrifugalization, the clear supernatant fluid was carefully pipitted off. The contents of the tubes were then rapidly desiccated in vacuum over calcium chloride at room temperature, then sealed and kept in the refrigerator. The sterility of the suspension was always determined by cultures. Usually all the cocci were dead in 24 hours. Only one streptococcus with which we have worked failed to be killed in 48 hours. The suspension of this latter organism became sterile only after 72 hours. When the killed organisms were to be injected, they were suspended in 2 or 3 c.c. sterile salt solution.

Numerous estimates of the numbers of cocci in such growths have shown that the average growth on a slant of blood agar after 24 hours comprises approximately 1,000,000,000 cocci.

When the streptococci were killed by heat, they were washed from the blood agar with 0.85 per cent sodium chloride solution, and the suspension heated to 60° for 30 minutes and kept in the ice-box.

*Effects of the injection into rabbits of streptococci killed by galactose solution.*—Rabbits were given one or more subcutaneous injections of streptococci killed by galactose, and examined as to the effects upon the opsonic index and as to subsequent resistance to living streptococci of the same strain as that from which the killed cocci were obtained. In no case did the injections produce any appreciable effect upon the animal other than a slight induration at the point of injection. This induration was not larger than a pea and was not accompanied by any inflammatory phenomena.

After galactose killed streptococci were injected into rabbits the opsonic index was found to follow a more or less regular course. A negative phase or fall in the index was inconstant and not usually pronounced. The fall was usually more marked after the primary than after subsequent injections. The index was usually highest on the second or third and fourth or fifth days after the injection. The character of the curve was similar with doses of varying size, but the larger doses usually produced higher indices.

When rabbits are injected subcutaneously with galactose killed streptococci, more or less immunity to the corresponding living streptococcus develops. After a single injection five to seven days

are required for this to appear. The protection afforded by two injections is greater than that following a single one. The accompanying tables will show the results obtained in representative instances:

TABLE 1.

Rabbit N, 1,220 grms.	Aug. 12, 1907. 1,000,000,000 galactose killed Streptococcus No. 3 subcutaneously	Aug. 17, 1907 2 blood-agar slants growth of Streptococcus No. 3 into peritoneal cavity	Died 4 days after inoculation
Rabbit O, 1,260 grms.	Aug. 13, 1907—do	do	Died 48 hrs. after inoculation
Rabbit P, 1,390 grms.	Aug. 14, 1907—do	do	Died 18 hrs. after inoculation
Rabbit Q, 1,120 grms.	Aug. 15, 1907—do	do	Died 22 hrs. after inoculation
Rabbit R, 1,420 grms.	Aug. 16, 1907—do	do	Died 48 hrs. after inoculation
Rabbit S, 1,410 grms.	Control	do	Died 18 hrs. after inoculation

In this experiment the killed streptococci were those from a single suspension equally divided. The living culture injected on August 17 consisted of the growth on 12 blood-agar slants, suspended in broth and divided into six equal parts.

TABLE 2.

Rabbit J, 1,600 grms.	July 30, 1907 500,000,000 galactose killed Streptococcus No. 3 subcutaneously Aug. 2, 1907 500,000,000 galactose killed Streptococcus No. 3 subcutaneously	Aug. 8, 1907 1 blood-agar slant growth of Streptococcus No. 3 into peritoneal cavity	Well a month after inoculation
Rabbit K, 1,500 grms.	July 30, 1907 500,000,000 galactose killed Streptococcus No. 3 subcutaneously Aug. 2, 1907 500,000,000 galactose killed Streptococcus No. 3 subcutaneously	do	Well a month after inoculation
Rabbit M, 1,710 grms.	Control	do	Died 18 hours after inoculation

In this experiment the killed streptococci were those from equal parts of a single suspension. The living cultures injected consisted of the growth from three slants of blood agar, suspended in broth, and divided into three equal portions.

TABLE 3.

Rabbit 1. 1,340 grms.	Aug. 29, 1908 1,000,000,000 galactose killed Streptococcus A. subcutaneously Sept. 3, 1908 1,000,000,000 galactose killed Streptococcus A. subcutaneously	Sept. 9, 1908 5 c.c. 24 hours broth culture Streptococcus A. intraperitoneally	Sept. 10 Slightly sick; Sept. 11 quite well; well a month later
Rabbit 2, 1,250 grms.	Control	Sept. 9, 1908 5 c.c. 24 hours broth culture Streptococcus A. intraperitoneally	Died 15 hours after inoc- ulation
Rabbit 3, 1,540 grms.	Oct. 5, 1908 500,000,000 galactose kill- ed Streptococcus A. subcutaneously	Oct. 15, 1908 0.2 c.c. 24 hrs. broth culture Streptococcus A. subcutaneously in ear	Oct. 16: Ear red and a little swollen Oct. 18: Ear much swollen —acts sick Oct. 19: Worse Oct. 20: Slightly better Oct. 21: Ear better. General condition much improved Oct. 22: Died
Rabbit 4, 1,765 grms.	Control	Oct. 15, 1908—do	Oct. 16: Ear red and a little swollen Oct. 18: Died

*Comparison of the effects of the injection into rabbits of streptococci killed by galactose solution and by heat.*—In testing the relative effect

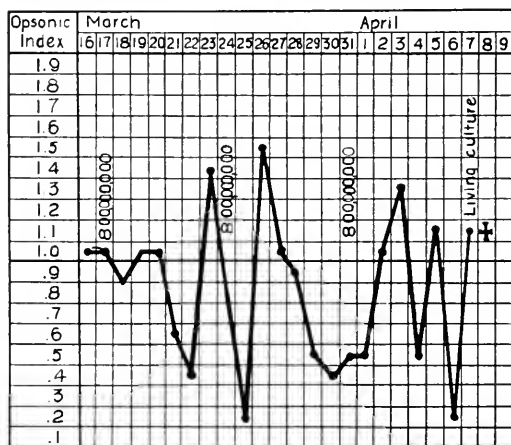


CHART 1.—Streptococco-opsonic index in Rabbit 1, Table 4, with approximate number of bacteria injected.

in rabbits of streptococci which were killed by suspension in 25 per cent galactose solution and by heat, individual animals were injected subcutaneously with equal amounts of the same culture of streptococci killed in each of the two ways. Those killed by heat were suspended in 0.85 per

time had elapsed to allow some degree of immunity to have developed, the animals of the series and a control animal were injected with equal amounts of a culture of the same streptococcus as that from which the killed organisms were obtained. Determinations of the opsonic index were also made in the rabbits during the experiment.

The following tables show the results obtained in three experiments. Accompanying these tables are charts of the opsonic index in the individual animals of the series:

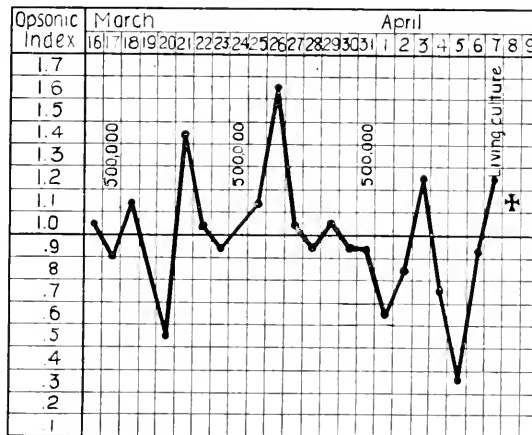


CHART 2.—Streptococco-opsonic index in Rabbit 2, Table 4, with approximate number of bacteria injected.

TABLE 4.

Rabbit 1, 1,040 grms.	Mar. 17, 1908 800,000,000 Streptococcus J. K. 13, killed by heat, subcutaneously Mar. 24, 1908—do Mar. 31, 1908—do	April 7, 1908 8 c.c. 20 hours broth culture Streptococcus J. K. 13, intraperito- neally	Died in less than 24 hrs. after inoculation
Rabbit 2, 1,160 grms.	Mar. 17, 1908 500,000 Streptococcus J. K. 13, killed by heat subcutaneously Mar. 24, 1908—do Mar. 31, 1908—do	do	Died in less than 24 hrs. after inoculation
Rabbit 3, 940 grms.	Mar. 17, 1908 800,000,000 galactose kill- ed Streptococcus J. K. 13, subcutaneously Mar. 24, 1908—do Mar. 31, 1908—do	do	Died between 2 and 3 days after inoculation
Rabbit 4, 1,200 grms.	Mar. 17, 1908 500,000 galactose killed Streptococcus J. K. 13, subcutaneously Mar. 24, 1908—do Mar. 31, 1908—do	do	Died between 1 and 2 days after inoculation

Unfortunately the control rabbit for this experiment received at least part of its injection of the living cultures in the abdominal wall

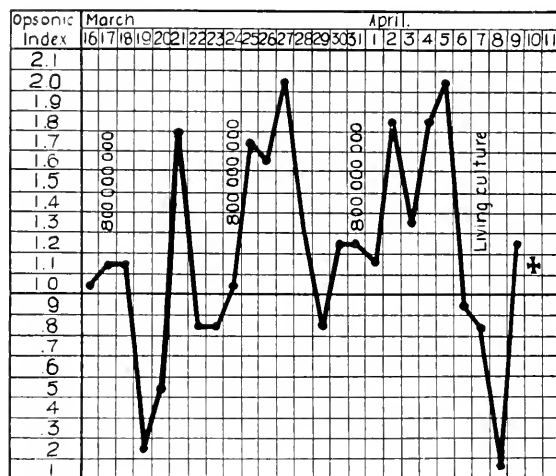


CHART 3.—Streptococco-opsonic index in Rabbit 3, Table 4, with approximate number of bacteria injected.

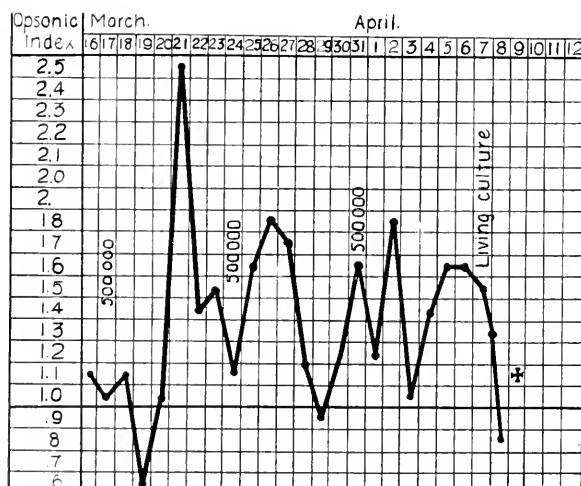


CHART 4.—Streptococco-opsonic index in Rabbit 4, Table 4, with approximate number of bacteria injected.

where a marked local swelling developed, but the animal lived.

TABLE 5.

Rabbit 1, 2,300 grms.	Sept. 22, 1908 1,000,000,000 heat killed Streptococcus A. sub- cutaneously Sept. 26, 1908—do	Oct. 2, 1908 5 c.c. 24 hrs. broth culture Streptococcus A. intra- peritoneally	Very sick 1½ hrs. after in- oculation Died in 8 hrs. after inoc- ulation
Rabbit 2, 2,190 grms.	Sept. 22, 1908 1,000,000,000 galactose killed Streptococcus A. subcutaneously Sept. 26, 1908—do	Oct. 2, 1908—do	Not sick 1½ hrs. after in- oculation Died 14 hrs. after inocu- lation
Rabbit 3, 2,100 grms.	Control	Oct. 2, 1908—do	Very sick 1½ hrs. after inoculation Died in 8 hrs. after inoc- ulation

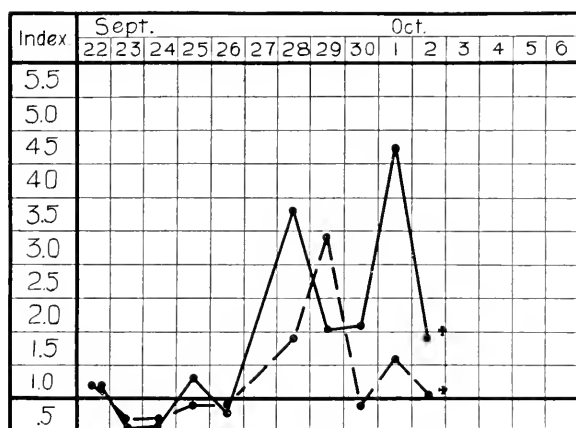


CHART 5.—Solid line, streptococco-opsonic index in Rabbit 2; broken line, streptococco-opsonic index in Rabbit 1, Table 5.

The dose of living streptococcus in this experiment was massive. One c.c. of the same culture killed a 2,000-gram rabbit in two days. If a somewhat smaller dose had been used, it is likely that more difference between the resistance of the animals would have been observed. The experiment is introduced as the opsonic curves are very typical.





From a study of the observations related above certain conclusions seem apparent. The subcutaneous injections of streptococci killed in galactose solution are followed by very definite phenomena. The serum of rabbits after such injections contain considerable amounts of opsonin which render the corresponding virulent streptococci susceptible to phagocytosis. Hand in hand with this rise of the opsonin, there develops in the rabbit a considerable degree of immunity to the living virulent streptococcus. The immunity thus acquired is sufficient to protect the experimental animal against such doses as kill acutely normal animals. The protection may be complete with a given dose, or may delay and modify the infection.

In marked contrast to this are the effects noticed after the injection into rabbits of streptococci killed by heat. Such rabbits do not produce any pronounced amount of opsonin for streptococci, and when injected with living cultures seem to have less resistance than normal rabbits.

#### EFFECTS OF THE INJECTION INTO MAN OF STREPTOCOCCI KILLED BY GALACTOSE SOLUTION.

Having found that streptococci killed by immersion in strong solution of galactose were without toxic effect in rabbits, it was decided to determine the results of such injections in man. Streptococci to the number of 500,000,000 killed in this way were injected in a healthy person beneath the skin. Following the injection there was no local reaction observed. Daily estimates of the opsonic index for the streptococcus, pneumococcus, and staphylococcus (*aureus*) were made, and the findings are shown in the accompanying chart (Chart 7). There is seen to be a double rise in the opsonic index for the streptococcus with no initial negative phase. The index for the pneumococcus and staphylococcus remained within normal limits.

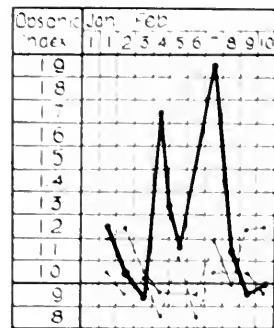


CHART 7. Opsonic indices in a healthy individual after a single injection of 500,000,000 streptococci killed in galactose solution, heavy solid line for streptococcus, lighter solid line for staphylococcus, broken line for pneumococcus.

INJECTIONS OF HOMOLOGOUS STREPTOCOCCI KILLED BY GALACTOSE  
SOLUTION IN THE TREATMENT OF STREPTOCOCCUS  
INFECTIONS IN MAN.

Encouraged by the results obtained in animals, injections of streptococci killed by galactose solution were employed in the treatment of patients with chronic streptococcus infections. The results have been so gratifying that the history of two such cases in which the opsonic index curve is complete are here reported. In each case the streptococcus employed was obtained from the patient to be treated, and the material for injection was prepared in the same manner as that used in the experiments on rabbits.

*Case I.*—Boy, age 5 years. Post-scarlatinal suppurative otitis media and mastoiditis.

June 5, 1907: Eleven days previously the boy had been discharged from Cook County Hospital after recovery from a moderately severe attack of scarlatina. Six days ago the cervical glands began to swell, and later an abscess in the neck opened spontaneously. At this time a discharge from the right ear made its appearance. Today on admission to the hospital there is a purulent discharge from the right ear, the cervical glands on the right side beneath the jaw are swollen and tender. On the left side of the neck there is a swelling as large as a walnut from which pus exudes through a narrow sinus.

June 11: Signs of mastoid involvement on the right side.

June 14: The mastoid opened and much purulent fluid evacuated; profuse purulent discharge has continued from the right ear.

June 24: Profuse, foul, discharge from right ear and mastoid wound.

July 9: Profuse discharge continues from both sources. The temperature has been irregular, running from 99° to 104° F. The pulse has become irregular and the general condition of the patient is bad.

July 17: 250,000,000 of galactose killed streptococci injected subcutaneously. The streptococcus was obtained from the pus from the mastoid wound, which yielded almost a pure growth of typical hemolyzing colonies in blood-agar plates.

Following the injection the temperature became normal in two days, and never again rose above 99.6° F. The general condition of the

patient also began to improve and the improvement was progressive. Injections were made on July 22 of 500,000,000; on July 29 of 250,000,000; and on Aug. 9 of 250,000,000 galactose killed streptococci. The accompanying chart (Chart 8) shows the course of the case with opsonic-index determinations. In this case the tendency had been to chronicity and the patient was constantly growing worse until the injections were begun. After the injections were begun the patient immediately began to improve generally, and the local condition in the ear and mastoid also became better. The improvement

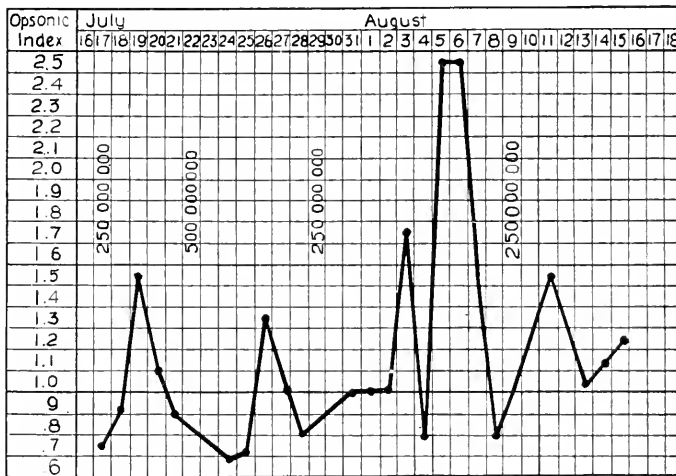


CHART 8.—Streptococco-opsonic index in Case I, p. 598, with approximate number of bacteria injected.

was especially rapid and marked when the index was highest on August 4 to 8.

*Case II.*—Boy, age 6 years. Chronic erysipelas with recurring acute attacks.

May 27, 1907: Upon this date the patient was admitted to the Home for Destitute Crippled Children because of a tuberculous spondylitis with associated discharging sinuses in the thigh. At this time there was a general swelling of the soft tissues of the face, being most marked about the eyes and nose. As to the previous history it was only possible to learn that the condition in the face had existed for a considerable time.

June 6: The boy had a chill with fever. A red spot appeared on one side of the nose, and the redness with swelling rapidly spread over the face. By June 10, when he was transferred to the contagious wards of Cook County Hospital, his condition was as follows: The face was enormously swollen and edematous; the eyes closed by the swelling. The skin was indurated, hard, reddish. The redness and swelling were sharply outlined, and included the external ears and forehead. There was considerable epiphora. The conjunctivae were injected. The temperature was 104° F. per rectum.

For a week the acute symptoms continued with an irregular temperature ranging from 102 to 105° F., and a pulse from 100 to 130. There was a profuse sero-purulent nasal discharge and double suppurative otitis media developed. The patient was very irritable and at times actively delirious.

From June 18 to July 19 the temperature was normal except for an occasional rise to 99 or 100° F. During the sickness repeated examinations failed to disclose albumen in the urine. Profuse purulent discharge from the sinuses in the thigh was present during all this period.

July 19 he was discharged from the hospital and returned to the Home for Crippled Children. At this time all acute symptoms were absent, but there was still present considerable swelling of the face. From July 19, 1907, to January 17, 1908, when the first injection of dead streptococci was given, his condition was constantly as follows: The skin of the face, including the ears and forehead, was much swollen, firm, waxy white. The thickening was most marked over the bridge of the nose, the upper lip, and lower eyelids. The swelling of the eyelids was often so severe as to prevent the opening of the eyes. There was constant epiphora from occlusion of the nasal duct. The conjunctivae were reddened and one cornea presented an opaque area. The nasal mucous membrane was much swollen; the nasal passages admitted only a small applicator. From the nose there was a constant sero-purulent discharge giving rise to excoriation of the skin of the upper lip. Cultures from the nasal secretions gave large numbers of streptococci, which produced hemolyzing colonies in blood-agar plates. There were few other bacteria present. The sinus in the thigh almost closed at times, but with

the recurrences in the face the discharge always became more profuse. Cultures from the sinus discharge yielded large numbers of hemolyzing streptococcus colonies, almost in purity.

The accompanying photograph was taken at a time when the face was at its best, and shows the general swelling of the tissues.



FIG. 1.

At intervals of four or five weeks there was added to the above picture the signs of an acute erysipelas. This began at the side of the nose and extended more or less widely. With such recurrences the temperature, otherwise normal, became elevated, and the patient became generally ill. These recurrences varied in severity, some

being mild, and one being as severe as that related early in the history when the patient was in the Cook County Hospital. This second very severe attack occurred in the first part of December, 1907, and was associated with unilateral otitis media. From the discharge from the ear blood-agar plates were prepared in which only hemolyzing colonies of streptococci developed. The culture of streptococcus obtained from the aural discharge was used for the later injections.

January 17, 1908: In the absence of acute symptoms, the patient was given subcutaneously in the well thigh 500,000,000 streptococci killed with galactose solution. The accompanying chart gives the course of the index after the injections (Chart 9). No local reaction followed the injection, except a slight deep induration, which was not tender to touch and disappeared after six days.

January 22: There was a mild recurrence with limited redness of the face and increased discharge from the sinus of the thigh.

January 24: The patient was given 250,000,000 streptococci killed with galactose solution, subcutaneously. Very slight deep induration followed the injection as before.

January 31: The face was more swollen and the skin was red about the nose. 250,000,000 streptococci killed with galactose solution were injected.

February 1: A moderately severe recurrence occurred, associated with profuse discharge from the sinuses in the thigh.

February 6: The patient had returned to his usual condition.

February 11: 500,000,000 streptococci killed with galactose solution were given. For about a week he seemed to do very well and on February 18 a mild recurrence occurred, mostly limited to one side of the face. From this time on he improved rapidly and continuously.

March 16: There was no swelling of the face with the exception of slight thickening of the upper lip and slight edema of the lower eyelids. Temperature normal. Sinus almost closed. Patient looks and feels well.

March 30: Boy seemed very well. One sinus in thigh discharged a little. There was no swelling of the face. The tissues were soft and free from edema. The nostrils were wide open, and there was slight nasal discharge.

April 21: Was very well. Had gained in weight and general con-

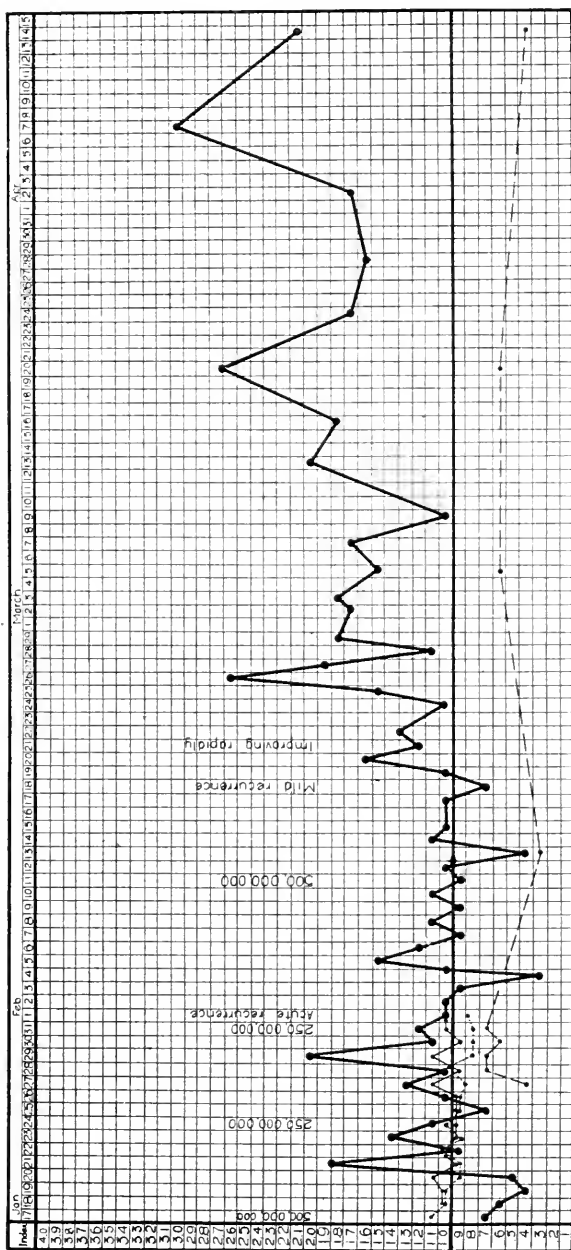


CHART 9.—Opsonic indices in Case II, p. 599; heavy solid line for streptococcus, lighter solid line for *Staphylococcus albus*; heavy broken line for tubercle bacillus, lighter broken line for pneumococcus. The figures represent the approximate number of streptococci injected.

dition was much improved. No swelling about the eyes. Conjunctivae appeared normal. Nostrils wide open. Temperature normal. The bridge of the nose and the upper lip are still slightly thicker than normal. One sinus in the thigh discharged a few drops daily. No streptococci in cultures from this discharge. The patient's father now insisted on taking the boy from the hospital and for some time he was lost track of. October 1 he was found by a visiting nurse and she reported that he had had no trouble in the interval and that his face looked perfectly normal.

In this case no pronounced improvement occurred until about a month after the first injection. During this period the opsonic index fluctuated from a little above to a little below normal. From the time the improvement in the condition of the face began and was progressive, the opsonic index remained constantly above normal. Each injection caused a fall in the streptococcus opsonic index, followed by a rise. After each injection the leucocytes increased, corresponding to the fall in the index; with the rise in the index the leucocytes decreased.

Following the first two injections the indices were estimated daily with respect to staphylococcus (*albus*) and the pneumococcus as well as the streptococcus. As shown in the chart with a varying streptococcus-opsonic index, the index for staphylococcus and pneumococcus constantly remained practically normal. The tuberculo-opsonic index was found below normal whenever estimated, varying from 0.4 to 0.7.

From these observations it would appear that the opsonic index for the streptococcus rose with the formation of anti-streptococcus bodies, and that the constantly elevated index corresponded to a considerable degree of immunity. The rise and fall of the index following the injections indicated the reaction of the body to the substances introduced; but only when the index stayed permanently above normal did the clinical picture indicate that the body was able to cope with the long infecting streptococci.

In each of the cases just described the streptococcus infection had been present for a considerable time, and the reactions of the body seemed unable to rid it of the infecting organisms. In Case II after each acute attack of erysipelas there was a stage of relative immunity,



but it never reached a degree sufficient to overcome the chronic infection in the tissues in and about the nose, and as this relative immunity wore off an acute attack again recurred.

These cases seem so convincing as to the therapeutic value of the injections of homologous streptococci killed by chemically indifferent agents that such injections are recommended in all cases of chronic streptococcus infection. It must be remembered that marked results may not occur until several injections have been administered, and a temporary aggravation of the clinical signs may follow an injection for a short time. The improvement appears only after a considerable degree of immunity has been established. The only available means of measuring immunity against the streptococcus is the estimation of the opsonin in the serum. The observations here recorded seem to indicate that the amount of opsonin in the blood serum is a valuable index of the degree of immunity, although of course other immune bodies may also be present.

In view of the results obtained in efforts to protect rabbits against virulent streptococci by injections of streptococci killed by heat, it is doubtful if it is of any advantage to inject patients with streptococci killed in this manner.

It may not be amiss to insist upon the necessity of the study of each pathogenic bacterium after it has been killed in various ways to determine the effects of its injection in suitable animals as to the production of immune bodies. This should in every case precede the use of the so-called "vaccine" in man. No general conclusions can safely be drawn from the study of one variety of bacterium as to the properties and activities of others.

Owing to difficulty in securing rabbits, no study has been made of the protective<sup>†</sup> action of injections of galactose killed streptococci of one strain against other strains. In the absence of observations on this point the use of heterologous streptococcal "vaccines" cannot be said to have any good experimental basis.

Gabritschewsky<sup>†</sup> and several other observers, mostly Russian, have employed injections of killed streptococcus cultures to produce immunity against scarlatina. Gabritschewsky used broth cultures, heated to 60° C. and preserved with 0.5 per cent phenol. Following

<sup>†</sup> *Centrallbl. f. Bakt., Orig.*, 1906, 41, pp. 719, 844; *Berl. klin. Wchnschr.*, 1907, 44, p. 556.

the injection of such material they have observed an erythematous eruption, a slight rise in temperature, angina, and sometimes vomiting. These symptoms do not appear in children who have passed through scarlatina, and after children have been subjected to these injections they do not develop scarlatina even though not isolated from those having clinical scarlet fever. With a great variability in susceptibility to scarlatina among children and with great difficulty in adequately controlling observations, it is not surprising that much skepticism should exist regarding the value to be attached to these observations.

It is desirable, however, that these observations should be repeated under circumstances where proper control as to exposure, etc., is possible. In such cases it may be suggested that streptococci isolated from cases of acute scarlatina be injected after being killed by galactose or other chemically indifferent agents, since this can be done without apparent injury to the person injected.

#### CONCLUSIONS.

Injections into rabbits of streptococci killed by suspension in 25 per cent galactose solution gives rise to a greater or less degree of protection against the subsequent injection of homologous living virulent organisms.

Such injections are followed by a marked increase in streptococco-opsonin, and a persistently high index may be taken to indicate a condition of immunity.

Whether the amount of opsonin is a measure of the full degree of immunity cannot be stated.

Injections into rabbits of streptococci killed by heat do not protect them against the subsequent injection of homologous living virulent organisms, but may even lower their natural resistance.

Such injections are not followed by any considerable increase in streptococco-opsonin.

The results of the injection in rabbits of streptococci killed by heat may throw some light upon the questionable results obtained by the injection of heat-killed streptococci in man in cases of streptococcal infection.

In cases of subacute and chronic infection by streptococci in man, therapeutic injections of homologous streptococci killed by chemically

indifferent agents are recommended. The use of streptococci killed by heat in such cases is of at least doubtful benefit.

In the case of every pathogenic bacterium the immunizing effect of the bacteria killed in various ways should be studied in animals and injections of such materials were better employed in man only when substantial favorable results have been observed in animals.

Until killed heterologous streptococci are shown to be active against various strains of streptococci, injections of homologous streptococci are preferable.

It is desirable to study the injection in children of streptococci isolated from cases of scarlet fever and killed by chemically indifferent agents with respect to its protective effects against scarlet fever.

## THE INJECTIONS OF HETEROLOGOUS STREPTOCOCCI, KILLED BY GALACTOSE, IN ERYSIPELAS AND IN SCARLET FEVER.\*

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IN view of apparently favorable results obtained by Weaver and Tunnicliff (p. 589), in cases of subacute and chronic infections by streptococci in which injections of homologous streptococci, killed by suspension in 25 per cent solution of galactose, were employed, it seemed desirable to try the effects of similar injections in a larger series of cases. Cases of erysipelas and scarlatina were selected for this study. Since it was necessary to make the injections as soon as possible after the cases came under observation, mixtures of streptococci from similar cases previously isolated and killed were used. The streptococci were treated in the manner described in the article mentioned. Special acknowledgment is made of the courtesy of Dr. Baum and Dr. Cameron, who kindly allowed the use in the Cook County Hospital of their cases for this study.

### THE EFFECTS OF THE INJECTION OF POLYVALENT HETEROLOGOUS STREPTOCOCCI KILLED BY SUSPENSION IN 25 PER CENT GALACTOSE SOLUTION IN CASES OF ERYSIPELAS.

The streptococci employed in the cases of erysipelas were obtained as follows: Pure cultures of typical hemolyzing streptococci were obtained from the nasal secretions of three cases of typical uncomplicated erysipelas and from the purulent discharge from a wound of the face complicated by typical spreading erysipelas. Equal quantities of streptococci from the four strains grown upon blood agar were suspended in 25 per cent galactose solution until the suspensions were sterile, when the suspension was distributed in small test-tubes, the clear fluid pipetted off after thorough centrifugalization, and the sediment rapidly dessicated in vacuum. The tubes were then sealed and kept in the ice-box until used. For injection the bacteria were

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suspended in 0.85 per cent salt solution. The dose injected contained about 200,000,000 killed streptococci.

Twenty-two cases of erysipelas received injections. Of these two were relapsing, six migrating, and three recurrent; of the rest, three were mild, four moderate, and six severe. Four involved the leg, and 18 the face. In all but three cases a single injection was given. In one case two injections were given, in one three injections, and in one recurrent case injections were given in several attacks.

Owing to the irregular course of the disease and the numerous clinical types met with, all of which may closely resemble each other in the initial stages, it is very difficult to judge of the effects of treatment. In only three cases of the series did the treatment appear to have any desired effect. All three were of the "migrating" type, one of the leg and two of the face. In all the spreading stopped within two days, and the patients made a rapid recovery. No opinion could be formed as to any action the treatment might have in preventing complications, as only four cases of abscess were met with, two in injected and two in uninjected cases. No other complications occurred. In the three cases that were known to be recurrent, the treatment did not prevent later recurrences. One case, which received several injections, had five attacks of typical facial erysipelas during eight months, the first attack lasting six weeks. Bacteriological examination during the first attack showed the nasal secretions to contain very few streptococci, and during the last two attacks streptococci could not be cultivated from the nasal secretion nor from the contents of vesicles developing during the course of the disease.

At the site of injection slight tenderness for 24 hours was sometimes observed, but no other local or systemic effect was noted. Systematic determinations of the opsonic index were not made. In one case the index rose from 1.0 on the day of the injection, to 4.0 on the third day following. In another case there was a rise from 1.0 to 2.1. In two cases the index seemed unaffected.

From our experience in these cases we are forced to conclude that the injection of killed polyvalent heterologous streptococci during the acute stage of erysipelas is without apparent effect upon the course of the disease, the cases doing no better than the controls which received no injections. That this would have been the case might

have been predicted since it was shown by Weaver and Tunicliff that in rabbits several days are required for immunity to appear following the injection. By the time such immunity has developed the patient's body may already have developed the immune bodies and gone through the reactions that led to recovery in the natural course of the disease.

In the three cases of the "migrating" type in which the course was more prolonged, the injections appeared to have a beneficial influence. The recurrent cases were under observation only until recovery from the acute attacks was complete. It is not unlikely that several injections with increasing doses would finally stimulate a sufficient degree of immunity to prevent further recurrence in such patients. In these cases it would be desirable to use streptococci isolated from the patient.

THE EFFECTS OF THE INJECTION OF POLYVALENT, HETEROLOGOUS  
STREPTOCOCCI KILLED BY SUSPENSION IN 25 PER CENT  
GALACTOSE SOLUTION IN CASES OF SCARLATINA.

The streptococci employed in the cases of scarlatina were isolated from the tonsils in acute cases, from purulent secretions from scarlatinal otitis media, from the pus in suppurative mastoiditis, and from the pus of suppurating cervical glands developing during the course of scarlatina. The growths from various cultures were mixed and prepared as in the case of erysipelas.

The investigation was conducted in connection with the cases admitted in the hospital during the months of April to September, 1908. In all 274 cases were under observation, of which 116 received the injections and the remaining 158 were considered as controls. The cases which received the treatment were selected at random, and not more than half the cases admitted during any one month were injected. While it was not expected that such injections would materially alter the course of the disease it was hoped that they would favorably influence the occurrence or duration of complications due to pyogenic organisms.

*Cases injected during the acute stage.*—Eighty-eight cases were injected during the acute stage of the disease before the occurrence of any purulent complications, and were studied with reference to the possible effect of the injections in preventing such complications.

Complications here considered are purulent rhinitis, otitis media, mastoiditis, severe cervical adenitis (suppurative and non-suppurative), and arthritis.

*Results.*—The following summary shows the number of cases with the most common complications and the percentages which they constitute of the entire number of each group:

TABLE 1.

	INJECTED		NOT INJECTED	
	No. of Cases	Per Cent	No. of Cases	Per Cent
Rhinitis.....	7	8	28	18
Otitis media.....	12	13.6	16	10
Cervical adenitis.....	5	6	16	10
Cervical abscess.....	1	1	5	3
Total cases*.....	20	23	48	31

\* The total number of cases does not correspond to the sum of those showing various complications since some cases had more than one complication.

This table seems to indicate, in the injected cases, a reduction in the percentage of all of the complications except otitis media, which, however, is by far the most frequent and important. But the series, as they stand, are not strictly comparable as shown in the following summary of the total number of cases, classified according to their severity, and the percentage of each series thus falling in each class.

TABLE 2.

	MILD		MODERATE		SEVERE		TOTAL CASES
	No. of Cases	Per Cent	No. of Cases	Per Cent	No. of Cases	Per Cent	
Injected.....	37	42	44	50	7	8	88
Not injected.....	73	48	65	41	20	13	158

Thus the control cases are shown to have an unduly large proportion of severe cases, which would, in part at least, account for the higher percentage of complications, since the largest number of complications is found in the severe cases as shown by the next summary in which the complicated cases are classified according to the severity of the original disease, the figures giving the percentage of cases in each class.

TABLE 3.

	MILD		MODERATE		SEVERE		TOTAL CASES
	No. of Cases	Per Cent	No. of Cases	Per Cent	No. of Cases	Per Cent	
Injected.....	3	8	13	30	4	57	20
Not injected.....	15	20	20	31	13	65	48

But there are two other factors which tend to distort results. A certain number of cases which were not injected when they came in developed complications later, and were then injected, and considered as "late injected" cases. Thus the non-injected cases, as they stand, do not represent the normal, but are a selected group of cases from which a few (16) complicated cases have been taken. Furthermore, a certain number of cases were already complicated when first seen and hence could not be included among the "injected early" group, but it would not be fair to include these among the controls, since they are a group in which it would be quite impossible to test the prophylactic properties of the injections. Revising these figures, then, so as to omit all cases showing complications during the first week of the disease, and adding to the control cases those that were not injected until late in the disease, we have the following:

TABLE 4.

	No. of Cases	Per Cent
Mild.....	74	48
Moderate.....	67	42
Severe.....	16	10
Totals.....	157	100

The revised figures to show the total number of complications and percentages of cases in each class which became complicated are given in Table 5.

TABLE 5.

	No. of Comp.	Per Cent
Mild.....	16	22
Moderate.....	18	27
Severe.....	9	56
Totals.....	43	27

Thus comparing Table 4 with Table 2 it is seen that the disproportion in the number of severe cases is so reduced as to make the



two series fairly comparable; and comparing Table 5 with Table 1 it is seen that the difference in the percentages of total complications in the two series is so small as to be negligible. The only effect of the injections seemed to be that in those injected cases in which complications arose they occurred on an average of eight days later than in the control cases.

From these results we may conclude that the injection of killed streptococci during the acute stage of scarlatina does not lead to the production of any considerable amount of antistreptococcus immunity. It may be that during this stage the capacity of the body to produce antibodies has been reduced by the scarlet fever infection.

*Cases injected after complications were present.*—Injections in cases in which complications were already present yielded much better results. Thirty-one cases were so treated, a few of them being injected more than once, and 11 (36 per cent) showed prompt improvement. The effect was much more marked in the subacute and chronic conditions than in the acute ones. Of nine cases injected during the first week of the fever, only one showed any immediate improvement. Of 23 cases injected later, 10 (44 per cent) showed prompt improvement. Of 18 cases injected during the first week of the complication eight (33 per cent) showed prompt improvement. Of 16 cases injected later seven (44 per cent) showed prompt improvement. The improvement was shown in a sharp reduction in the amount of discharge (from ears, nose, or cervical abscess), in from two to six days after the injection. In some instances the discharge would cease completely in this time, and in others a very slight discharge would continue for a week or two, then cease. In two instances a nasal discharge which had resisted local treatment for two weeks, had ceased completely five days after injection. In one case an aural discharge which had lasted for more than a month without any indication of improvement stopped completely two days after the injection. Such instances of rapid improvement while occasionally met with in the uninjected cases were usually followed later by a more profuse discharge. One case had purulent discharge from ears, nose, and eyes, a pustular dermatitis as a result of the discharges, superficial ulcerations on lips and tongue, and a general septic condition. These had resisted local and general treatment, and the boy

was getting worse. Three days after an injection he showed a very marked local and general improvement. The evidences of sepsis had disappeared, the nasal and ocular discharges had ceased, the aural discharge was much less, and the skin lesions were healing rapidly, and six days after the injection the aural discharge had ceased completely. The ulcerations on the tongue, however, lasted two or three weeks.

In two or three cases a cervical gland suppurated soon after the injection but even if this result could be ascribed to the injection, the danger is remote because of the large number of similar cases in which no such result occurred. Aside from this, no bad result of any kind was observed as a result of the injections. In the treatment of the later streptococcus complications in scarlatina, it is not unlikely that better results would follow the use of homologous streptococci in preparing the material for injection, and it would be desirable to repeat the injections at intervals of five to seven days, the dose being increased from 200,000,000, to 500,000,000 or 800,000,000. In rabbits the larger doses seemed to produce more reaction and greater immunity, and it is probable that this would hold true in man. Too large initial doses are, however, to be avoided because of the temporary reduction in resistance which may follow and may explain the slight aggravation of discharges and other symptoms sometimes occurring on the day after injections, and the apparent hastening of suppuration in swollen glands in occasional instances.

#### CONCLUSIONS.

1. The injection of polyvalent, heterologous streptococci killed by chemically indifferent agents during the acute stage of erysipelas has no appreciable effect upon the course of the disease. In cases running a prolonged course such injections appear to exert a favorable effect.

2. The injection of such streptococci during the early stages of scarlet fever does not prevent the later development of local streptococcus complications, although they may appear a little later in the disease.

3. The injection of such killed streptococci after local streptococcus complications have developed in scarlatina exerts considerable in-

fluence in hastening recovery. The later the complications appear, the better the results following the injections.

4. Homologous streptococci are probably preferable for preparing the material for injection both in protracted, subacute, chronic, and recurring cases of erysipelas and in cases of scarlatina with local streptococcus infection.

# SIMPLE SYNTHETIC MEDIA FOR THE GROWTH OF *B. COLI* AND FOR ITS ISOLATION FROM WATER.\*

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THE purpose of this research was first, to prepare a simple synthetic culture medium for the growth of *B. coli*, and second, to find a simple synthetic medium to be used in water analysis, which would favor the growth of *B. coli* and would inhibit the growth of the other organisms commonly present in water.

The advantages of a synthetic medium are plain. As its chemical composition is exactly known it can be easily and quickly prepared, and can always be duplicated. Results obtained by its use are always uniform and comparable. By the use of such a medium some light may be thrown on bacterial metabolism.

## I. SIMPLE SYNTHETIC MEDIA FOR THE GROWTH OF *B. coli*.

Some experimental work has been done already with synthetic culture media for the growth of *B. coli*. Galimard and Lacomme<sup>1</sup> give the chemical composition of several media on which *B. coli* and certain other organisms grow well. They worked with amido acids as the bases of their media. One of their media had the following composition:

Leucin.....	7.5 gr.
Alanin.....	2.0 gr.
Tyrosin.....	traces
Glycerin.....	15 gr.
Sodium chloride.....	0.5 gr.
Magnesium sulphate.....	0.05 gr.
Calcium glycerophosphate.....	0.2 gr.
Sodium carbonate.....	to neutralize
Distilled water.....	1,000 c.c.

The following organisms were found to grow on this medium:

*B. pyocyaneus*  
*B. prodigiosus*  
*B. coli* R<sup>2</sup>  
*Pneumobacillus* of Friedlander  
*B. tetragenus*  
*Staphylococcus aureus*  
*Sarcina superba*

\* Received for publication October 20, 1908.

<sup>1</sup> *Jour. de physiol. et path. générale*, 1907, 9, p. 481.

*B. coli* was also grown on several other media similar to the above but containing other amido acids such as glycoll, aspartic acid, etc.

MacConkey<sup>1</sup> showed that *B. coli* and other intestinal bacteria grow well on an agar medium containing 0.5 per cent of sodium taurocholate, while the common bacteria are to a great extent inhibited.

Jackson<sup>2</sup> states that the bile salts, when added to a Standard agar medium, exert a strong restraining action on most species of bacteria which grow at blood heat. This restraining action is selective, it favors the growth of *B. coli*, retards the growth of certain streptococci, and actually kills off the majority of species which grow at 37° C. Jackson says that the effect must be due to the cholic acid radical which is common to both salts.

Both MacConkey's and Jackson's media have a very complex chemical composition and a variable one. Galimard and Lacomme's medium has the advantage of having a fixed chemical composition but is still too complex to be of practical value in water analysis. If their medium could be simplified and if the inhibitive property of Jackson's medium could be given to it we would have a medium of practical value.

As *B. coli* was known to grow on several synthetic media containing amido compounds the following was tried:<sup>3</sup>

Sodium chloride.....	0.5 gr.
Disodium phosphate.....	0.2 gr.
Ammonium lactate.....	0.6 gr.
Asparagin.....	0.4 gr.
Distilled water.....	100 c.c.
Sodium carbonate.....	to neutralize

*B. coli* freshly isolated from feces grew very well on this medium. After 12 hours at 37° C. there was a strong turbidity in the inoculated tubes; after 24 hours there was a heavy sediment at the bottom of the tubes and a pellicle at the surface.

Ammonium lactate was then omitted from the medium and the following tried:

<sup>1</sup> *Thompson Yates Laboratories Rep.*, 1900, 3, p. 41; 1901, 3, p. 151; MacConkey, A., and Hill, Chas. *ibid.*, 1901, 4, p. 151.

<sup>2</sup> *Biological Studies by the Pupils of William Thompson Sedgwick*, Boston, 1906, p. 292.

<sup>3</sup> In all cases throughout this work C. P. chemicals and water distilled in glass were used.

Sodium chloride.....	0.5 gr.
Disodium phosphate.....	0.2 gr.
Asparagin.....	1 gr.
Distilled water.....	100 c.c.
Sodium carbonate.....	to neutralize

*B. coli* also grew well on this medium. This medium contains but one organic compound, the other substances are all inorganic salts.

The next experiment was to determine whether *B. coli* can grow on amido compounds alone. The results are given in Table 1.

TABLE 1.

NUMBER OF MEDIUM	COMPOSITION OF MEDIUM	GROWTH OF <i>B. coli</i> AT 37° C.	
		In 24 Hours	In 48 Hours
1.....	Glycocoll 1 gr. Distilled water 100 c.c.	None	None
2.....	Glycocoll 1 gr. Sodium chloride 0.2 gr. Distilled water 100 c.c.	None	None
3.....	Glycocoll 1 gr. Potassium chloride 0.2 gr. Distilled water 100 c.c.	None	None
4.....	Asparagin 1 gr. Distilled water 100 c.c.	None	None
5.....	Urea 1 gr. Distilled water 100 c.c.	None	None

All made neutral to phenolphthalein with NaOH.

Under the above conditions *B. coli* does not grow on glycocoll, asparagin, or urea alone. These experiments are controls on the purity of our salts and of our distilled water and their freedom from the salts which we shall find later are required for the growth of *B. coli*.

The next experiments were tried to show what inorganic salts *B. coli* requires in addition to asparagin. The latter was selected as the organic base of the media not only because it was less expensive than the others but also because further experiments showed it to be the most favorable. The more common soluble inorganic salts were tried. The results are given in Table 2.

The experiments in Table 2 show that *B. coli* grows on a combination of asparagin with either sodium or ammonium nitrate or sodium or ammonium phosphate but grows much better on the phosphates than on the nitrates.

TABLE 2.

NUMBER OF MEDIUM	COMPOSITION OF MEDIUM		GROWTH OF <i>B. coli</i> AT 37° C.	
			In 24 Hours	In 48 Hours
6.....	Asparagin 1 gr. NaCl 0.2 gr. Distilled water 100 c.c.		None	None
7.....	Asparagin 1 gr. KCl 0.2 gr. Distilled water 100 c.c.		None	None
8.....	Asparagin 1 gr. NH <sub>4</sub> Cl 0.2 gr. Distilled water 100 c.c.		None	None
9.....	Asparagin 1 gr. CaCl <sub>2</sub> 0.2 gr. Distilled water 100 c.c.		None	None
10.....	Asparagin 1 gr. MgCl <sub>2</sub> 0.2 gr. Distilled water 100 c.c.		None	None
11.....	Asparagin 1 gr. Na <sub>2</sub> SO <sub>4</sub> 0.2 gr. Distilled water 100 c.c.		None	None
12.....	Asparagin 1 gr. K <sub>2</sub> SO <sub>4</sub> 0.2 gr. Distilled water 100 c.c.		None	None
13.....	Asparagin 1 gr. (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> 0.2 gr. Distilled water 100 c.c.		None	None
14.....	Asparagin 1 gr. NaNO <sub>3</sub> 0.2 gr. Distilled water 100 c.c.		Slight	Slight
15.....	Asparagin 1 gr. KNO <sub>3</sub> 0.2 gr. Distilled water 100 c.c.		None	None
16.....	Asparagin 1 gr. NH <sub>4</sub> NO <sub>3</sub> 0.2 gr. Distilled water 100 c.c.		Very slight	Slight
17.....	Asparagin 1 gr. Na <sub>2</sub> HPO <sub>4</sub> 0.2 gr. Distilled water 100 c.c.		Good	Very good
18.....	Asparagin 1 gr. K <sub>2</sub> HPO <sub>4</sub> 0.2 gr. Distilled water 100 c.c.		None	None
19.....	Asparagin 1 gr. (NH <sub>4</sub> ) <sub>2</sub> HPO <sub>4</sub> 0.2 gr. Distilled water 100 c.c.		Good	Very good

All made neutral to phenolphthalein with NaOH.

The next step was to determine whether the concentration of these salts has any influence on the growth and whether the combination of two salts would be an improvement.

The following table (Table 3) gives the results there enumerated. As far as could be noticed the increase in the concentration of the salts or the combination of two salts does not improve the growth.

TABLE 3.

NUMBER OF MEDIUM	COMPOSITION OF MEDIUM	GROWTH OF <i>B. coli</i> AT 37° C.	
		In 24 Hours	In 48 Hours
20.....	Asparagin 1 gr. NaNO <sub>3</sub> 0.2 gr. Distilled water 100 c.c.	Very slight	Slight
21.....	Asparagin 1 gr. NaNO <sub>3</sub> 0.4 gr. Distilled water 100 c.c.	None	No better than on 20
22.....	Asparagin 1 gr. NH <sub>4</sub> NO <sub>3</sub> 0.2 gr. Distilled water 100 c.c.	None	Very slight
23.....	Asparagin 1 gr. NH <sub>4</sub> NO <sub>3</sub> 0.4 gr. Distilled water 100 c.c.	None	No better than on 22
24.....	Asparagin 1 gr. Na <sub>2</sub> HPO <sub>4</sub> 0.2 gr. Distilled water 100 c.c.	Good	Very good
25.....	Asparagin 1 gr. Na <sub>2</sub> HPO <sub>4</sub> 0.4 gr. Distilled water 100 c.c.	Good; same as on 24	Very good; same as on 24
26.....	Asparagin 1 gr. (NH <sub>4</sub> ) <sub>2</sub> HPO <sub>4</sub> 0.2 gr. Distilled water 100 c.c.	Good	Very good
27.....	Asparagin 1 gr. (NH <sub>4</sub> ) <sub>2</sub> HPO <sub>4</sub> 0.4 gr. Distilled water 100 c.c.	Good; same as on 26	Very good; same as on 26
28.....	Asparagin 1 gr. NaNO <sub>3</sub> 0.2 gr. Na <sub>2</sub> HPO <sub>4</sub> 0.2 gr.	Good; same as on 24	Very good; same as on 24
29.....	Asparagin 1 gr. NH <sub>4</sub> NO <sub>3</sub> 0.2 gr. (NH <sub>4</sub> ) <sub>2</sub> HPO <sub>4</sub> 0.2 gr. Distilled water 100 c.c.	Good; same as on 26	Very good; same as on 26
30.....	Asparagin 1 gr. NaNO <sub>3</sub> 0.2 gr. (NH <sub>4</sub> ) <sub>2</sub> HPO <sub>4</sub> 0.2 gr. Distilled water 100 c.c.	Good	Good
31.....	Asparagin 1 gr. NaNO <sub>3</sub> 0.2 gr. NH <sub>4</sub> NO <sub>3</sub> 0.2 gr. Distilled water 100 c.c.	Slight	Good
32.....	Asparagin 1 gr. Na <sub>2</sub> HPO <sub>4</sub> 0.2 gr. (NH <sub>4</sub> ) <sub>2</sub> HPO <sub>4</sub> 0.2 gr. Distilled water 100 c.c.	Good	Good

All made neutral to phenolphthalein with NaOH.

We may decide then that either one of the two following media may be considered favorable for the growth of *B. coli* at 37° C. A good growth is obtained even in the first 24 hours.

## MEDIUM 1

Asparagin..... 1 gr.  
Na<sub>2</sub>HPO<sub>4</sub>..... 0.2 gr.  
Distilled water..... 100 c.c.

## MEDIUM 2

Asparagin..... 1 gr.  
(NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub>..... 0.2 gr.  
Distilled water..... 100 c.c.

Made neutral to phenolphthalein with NaOH.



2. THE USE OF THE ABOVE MEDIA FOR THE ISOLATION OF *B. coli* FROM WATER.

A. *Lactose-asparagin agar*.—Inasmuch as in the routine of water analysis a solid medium is required we next directed our attention to the combination of the simple media described, with agar, for the purpose of isolating the colon bacillus by the plate method.

The agar was purified by cutting it into small pieces and soaking in distilled water for 24 hours, and the following medium which may be called lactose-asparagin agar was prepared:

Purified agar (3 per cent solution).....	250	c.c.
Asparagin	2.5	gr. }
Na <sub>2</sub> HPO <sub>4</sub>	0.5	gr. }
Distilled water	250	c.c. }
		.....250 c.c.

Made neutral to phenolphthalein with NaOH and 1 per cent lactose added.

Plates were made using this lactose-asparagin agar and 1 per cent azolitmin<sup>1</sup> solution in the usual manner. Several samples of water slightly polluted with *B. coli* were plated with the azolitmin-lactose-asparagin agar. Numerous colonies developed in each plate after 24 hours at 37° C. Several red colonies were isolated and proved to be *B. coli*.

The above medium, judging from repeated trials, appears to be just as effective as the Standard litmus-lactose agar for the isolation of *B. coli* and besides it has the advantage of having a fixed and simple chemical composition.

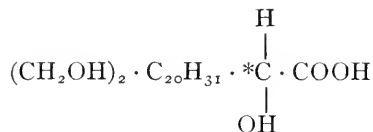
B. *The addition of certain substances to the above medium for the purpose of inhibiting the growth of the bacteria in water other than B. coli*.—Jackson has proved by experiments that the bile salts (sodium taurocholate and glycocholate) favor the growth of *B. coli* and restrain the growth of other forms of water bacteria. He proved that this restrictive action was due to the cholic group of the bile salts.

Sodium glycocholate and taurocholate could be added to the above medium to restrain the growth of other bacteria. But pure bile salts are too expensive to be used on a large scale. The use of

<sup>1</sup> There are several kinds of azolitmin on the market. Kahlbaum's C. P. azolitmin was used exclusively in these experiments. One gram was dissolved in 100 c.c. of distilled water and the solution boiled for 15 minutes. The solution which at first is slightly red turns to a blue after boiling. Prepared in this way the azolitmin solution does not need to be treated with an alkali to bring it to the right reaction. It may be used directly with any culture medium which is neutral to phenolphthalein. Other samples of azolitmin do not act in this way.

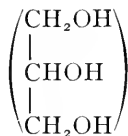
bile itself would defeat the object of these experiments as its composition is so complex and variable.

It was thought that very probably simpler and cheaper substances could be found which would have the same selective action. A consideration of the chemical structure of cholic acid led to the following: Cholic acid is



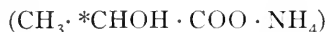
It has two primary alcohol groups ( $\text{CH}_2\text{OH}$ ), and one a symmetrical carbon atom indicated by a star (\*). Many substances could be selected having either an asymmetrical carbon atom, or the ( $\text{CHOH}$ ) group, or the ( $\text{CH}_2\text{OH}$ ) group, or all of them combined. The following substances were tried:

*Glycerin*



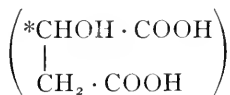
which has two primary alcohol groups and one secondary alcohol group.

*Ammonium lactate*



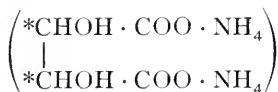
which has the secondary alcohol group and an asymmetrical carbon atom.

*Malic acid*



which has the secondary alcohol group and an asymmetrical carbon atom.

*Ammonium tartrate*



which has two secondary alcohol groups and two asymmetrical carbon atoms.

Experiments were then begun to determine whether these various substances could be substituted for the asparagin with the idea that if this is possible they might serve both as a food for *B. coli* and as inhibitory agents for the other organisms.

The results are given in the following table (Table 4):

TABLE 4.

NUMBER OF MEDIUM	COMPOSITION OF MEDIUM	GROWTH OF <i>B. coli</i> AT 37° C.		
		In 24 Hours	In 48 Hours	In 72 Hours
33.....	Glycerin 1 gr. (NH <sub>4</sub> ) <sub>2</sub> HPO <sub>4</sub> 0.2 gr. Distilled water 100 c.c.	Good	Very good	Very good
34.....	Ammonium lactate 1 gr. Na <sub>2</sub> HPO <sub>4</sub> 0.2 gr. Distilled water 100 c.c.	Good	Very good	Very good
35.....	Malic acid 1 gr. (NH <sub>4</sub> ) <sub>2</sub> HPO <sub>4</sub> 0.2 gr. Distilled water 100 c.c.	Slight	Slight	Slight
36.....	Ammonium tartrate 1 gr. Na <sub>2</sub> HPO <sub>4</sub> 0.2 gr. Distilled water 100 c.c.	None	None	None

All made neutral to phenolphthalein with NaOH.

These experiments were repeated several times with the same results; a good growth always occurred on media 33 and 34; a visible growth on 35, and no growth at all on 36.

Solid culture media were then made with the above solutions in the same way that the lactose-asparagin agar was prepared. A series of comparative tests was then conducted in the Laboratory of the Rhode Island State Board of Health. Samples of water known to be slightly polluted were plated on the ordinary Standard litmus-lactose agar and at the same time on azolitmin-lactose-asparagin agar, azolitmin-lactose-glycerin agar, azolitmin-lactose-ammonium lactate agar, and azolitmin-lactose-malic acid agar. The results appear in the following table (Table 5).

As will be seen from this table the results were very satisfactory. The red colonies were well developed and characteristic of *B. coli* on the four synthetic media. There were fewer red colonies on the synthetic media than on the Standard medium but the numbers

TABLE 5

Plate No.....		Standard Agar			Asparagin Agar			Glycerin Agar			Ammonium Lactate Agar			Malic Acid Agar		
		1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Pawtuxet River	Red colonies	5	2	2	5	0	2	0	1	0	0	2	0	0	0	1
Feb. 26, 1908	Total	20	18	14	12	8	2	0	2	0	0	4	2	0	0	1
Pawtuxet River	Red	18	16	15	3	0	0	1	3	0	1	1	2	0	0	0
Mar. 11, 1908	Total	24	22	20	10	9	13	1	3	0	4	3	6	0	0	0
Pawtuxet River	Red	10	6	7	3	2	3	2	4	4	1	2	2	2	0	1
Mar. 25, 1908	Total	14	10	14	5	4	4	2	4	5	1	2	2	2	0	1
Pawtuxet River	Red	10	15	13	2	3	2	3	3	3	3	3	3	2	0	1
April 8, 1908	Total	25	24	18	2	4	4	14	0	5	8	4	2	0	1	1
Ten Mile River	Red	2	2	1	1	1	0	0	2	1	0	0	2	1	0	0
Mar. 2, 1908	Total	16	18	14	14	21	16	12	14	24	8	20	4	3	0	0
Ten Mile River	Red	4	3	4	1	2	0	2	1	1	3	0	1	0	0	2
Mar. 18, 1908	Total	20	14	20	4	4	1	3	3	2	3	1	3	0	0	4

were always fairly proportional. Very few colonies other than red ones appeared on the glycerin, ammonium lactate, and malic acid plates. On the asparagin agar plates there were more colonies other than red ones than on the other synthetic media. On the malic acid agar there were never any other colonies than the red ones, and the red ones were themselves in smaller number. In this connection it is suggested that it might be possible in the case of slightly polluted water to use malic acid agar to plate a larger amount than 1 c.c. of the water to be tested. Experiments were not carried on along this line. No spreading colonies ever appeared on the four synthetic media while there were many on the Standard agar plates. Some of the red colonies on the Standard agar were not at all characteristic of *B. coli* while the red colonies on the four synthetic media were all of the *B. coli* type.

It was then thought advisable to prove whether all the red colonies on the four synthetic media were *B. coli*. All the red colonies were carefully fished from the plates made April 8, 1908, from the Pawtuxet River water. There were 38 red colonies fished from the three Standard agar plates as controls, seven from the three asparagin agar plates, nine from the three glycerin agar plates, nine from the three ammonium lactate agar plates and three from the three malic acid agar plates. Each one was run through the following tests for *B. coli*: (1) morphology; (2) agar slant; (3) gelatin stab; (4) fermentation of dextrose; (5) coagulation of milk; (6) reduction of nitrates.

The results are as follows:

TABLE 6.

Origin of the Colonies	No. of Red Colonies	No. of <i>B. coli</i> Colonies	Percentage of <i>B. coli</i>
Standard litmus-lactose agar.....	38	18	48
Litmus-lactose-asparagin agar.....	7	7	100
Litmus-lactose-glycerin agar.....	9	9	100
Litmus-lactose-ammonium lactate agar.....	9	9	100
Litmus-lactose-malic acid agar.....	3	3	100

The above table shows that of all the red colonies which developed on the Standard litmus-lactose agar only about half of them were *B. coli*, while all the red colonies which developed on the four synthetic culture media were true *B. coli*. The above table also shows that there were more *B. coli* per c.c. of water developing on the ordinary agar than on the synthetic media.

## SUMMARY.

1. *B. coli* does not grow on dilute (1 per cent) solutions of asparagin, glycocoll, or urea.
2. It grows very well on a 1 per cent solution of asparagin if 0.2 per cent of sodium phosphate is added.
3. Of all the soluble inorganic salts sodium and ammonium nitrate and sodium and ammonium phosphate are the only ones which can be used with asparagin for the growth of *B. coli*.
4. Substances having an asymmetric carbon atom in their molecule and a CHOH group, such as glycerin, ammonium lactate, malic acid, can be used instead of asparagin for the growth of *B. coli*.
5. These substances seem to favor the growth of *B. coli* and inhibit the growth of water organisms.
6. Very simple synthetic culture media can be used in place of the ordinary litmus-lactose agar or the various bile media and they have several advantages in the routine work of detecting *B. coli* in water.
7. Of the four synthetic media suggested two are especially recommended. Their composition is as follows:

## I

Purified agar (3 per cent solution) .....	500 c.c.
Glycerin 5 gr.	} .....
Ammonium phosphate 1 gr.	
Distilled water 500 c.c.	

Sodium hydroxide solution is used to neutralize and 1 per cent lactose is added just before sterilization.

## II

Purified agar (3 per cent solution)..... 500 c.c.  
Ammonium lactate 5 gr. }  
Disodium phosphate 1 gr. } ..... 500 c.c.  
Distilled water 500 c.c. }  
Sodium hydroxide solution is used to neutralize and 1 per cent lactose is added as before.

Both of the above media are to be used with 1 per cent azolitmin solution in the usual way.

8. These media have thus far given extremely satisfactory results for the isolation of *B. coli* in practical water analysis.

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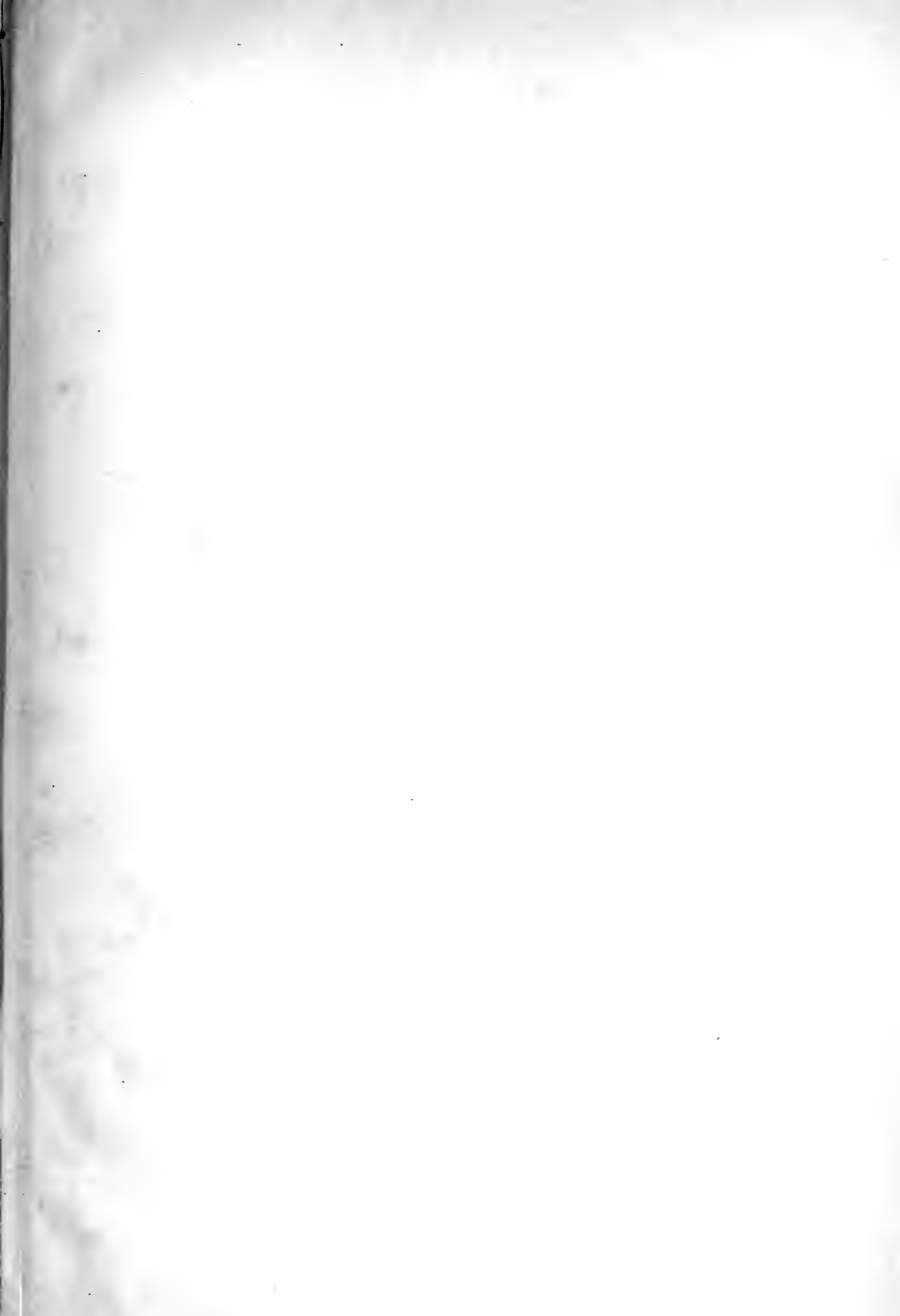
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